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GENETIC STUDIES IN DEVELOPMENTAL SKELETAL AND LIMB DEFECTS

Boyan Ivanov DIMITROV

Doctoral thesis in Medical Sciences

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Dedicated to my parents

CONTENTS

ABBREVIATIONS

ACTB	β-actin
AD	Autosomal Dominant
AER	Apical Ectodermal Ridge
AP	Anterior-Posterior
AR	Autosomal Recessive
B2M	β-2-microglobulin
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BLAT	BLAST-like Alignment Tool
BMP	Bone Morphogenic Protein
bp	base pair
cDNA	complementary DNA
CGH	Comparative Genomic Hybridization
CLK2	CDC-like Kinase 2
CNV	Copy Number Variation
dbSNP	database Single Nucleotide Polymorphism
DICEPHER	Database of Chromosomal Imbalances and Phenotypes in Humans using Ensembl Resources
DIRC1	Disrupted in Renal Carcinoma 1
DLDMS	Distal Limb Deficiency Micrognathia Syndrome
DNA	Deoxyribonucleic acid
DV	Dorsal-Ventral
EBV	Ebstain-Bar virus
ECARUCA	European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
ECM	exracellular matrix
ESDN	European Skeletal Dysplasia Network
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FISH	Fluorescent *in situ* Hybridization
FMN1	Formin 1
FoSTeS	Fork Stalling and Template Switching
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF6	Growth Differentiation Factor 6
GDF8	Growth Differentiation Factor 8
GEO	Gene Expression Omnibus
GREM1	Gremlin 1
GULP	PTB domain-containing engulfment adaptor

GUSB	β-glucuronidase
HOXA/ HOXD	Homeobox A/ D transcription factor
ISDS	International Skeletal Dysplasia Society
ITM2B	Integral Membrane protein 2B
Kb	Kilobase(s)
KFA	Klippel-Feil anomaly
LCR	Low Copy Repeat
LRCH	Leucine-rich repeats and Calponin Homology domain containing 1
LRRTM4	Leucine-rich Repeat Transmembrane neuronal 4
m-e	mesenchymal-ectodermal
Mb	Megabase(s)
MED4	Mediator complex subunit 4
MSD	Mesomelic Skeletal Dysplasia
NAHR	Non-Allelic Homologous Recombination
NAEJ	Non-Allelic End Joining
NCBI	National Centre for Biotechnology Information
OMIA	On-line Mendelian Inheritance in Animals
OMIM	Online Mendelian Inheritance in Man
ORMDL1	ORM1-like protein 1
p53	Transformation-related Protein 53
PCR	Polymerase Chain Reaction
PD	Proximal-Distal
PMS1	Postmeiotic Segregation increased 1
PSM	Paraxial pre-Somitic Mesoderm
RLD	Reduction Limb Defect
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT Q-PCR	Real Time Quantitative-Polymerase Chain Reaction
SALL3	Sal-like 3
SHFLD	Split Hand-Foot malformation with Long bone Deficiency
SHFM	Split Hand-Foot Malformation
SHH	Sonic Hedgehog Homolog
SNP	single nucleotide polymorphism
SUCLA2	Succinate-CoA ligase, ADP-forming, beta subunit
TBX5	T-box transcription factor 5
UBC	Ubiquitin C

UCSC	University of California Santa Cruz
UPD	Uniparental Disomy
UniGene	Unique Gene
VDRIP (=MED4)	Vitamin D Receptor Interacting Protein
WDR75	WD repeat domain 75
WNT	Wingless-Type
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta isoform
ZPA	Zone of Polarizing Activity

SUMMARY

The congenital skeletal and limb defects occur in 1 in 500 to 1 in 1000 births. As the limbs were a relatively recent developmental event in the evolution of vertebrates, many molecular pathways controlling the limb/ skeletal formation were co-opted from already existing patterning programs. Because of this genetic pleiotropy, more than 50% of affected individuals have associated inborn defects of other organs and tissues. The consequence of this relatively high frequency and severe, lifelong multiorgan burden is that these patients represent a substantial proportion of the consultations in the clinic and require a multidisciplinary approach, including not only medical but also several paramedical specialties like physiotherapists, psychologists and social workers. Thus, the congenital limb / skeletal defects are also of significant social importance.

Since many years the vertebral limb development has served as an important model to study morpho- and organo-genesis. Several spontaneous and genetically manipulated (transgenic) animal models contributed to the discovery of important signalling pathways involved in limb and skeletal formation. In addition, as several of these key genetic players are involved in other developmental cascades, this knowledge contributed significantly to the understanding of the patterning of other organs and systems. The enormous impact of this has been the development of new medical branches and therapeutic approaches.

Surprisingly, despite this importance in developmental biology and genetics, the genetic studies of congenital limb/ skeletal defects in man have been a relatively neglected topic. Therefore, by applying both different classical as well as new techniques, we aimed to increase the current knowledge regarding the causal molecular mechanisms involved in this group of congenital anomalies. In addition, we aimed to establish a systematic approach to clinical management and genetic analysis in order to arrive at a correct diagnosis. Last but not least, the consequence of this will be the opportunity to provide appropriate genetic counselling to affected individuals and their relatives and thus possibilities for more effective prenatal diagnosis.

For this reason, a group of patients with clinically and genetically unexplained complex skeletal phenotypes were clinically well characterized and sub-grouped for further research utilizing classical “forward genetic” and “reverse genetic” methodologies.

In the first part of this project, we analyzed, at molecular level, two balanced chromosomal rearrangements (*de novo* translocation and inversion) associated with the congenital skeletal abnormalities, Mesomelic Skeletal Dysplasia and Klippel-Feil syndrome. The precise breakpoint mapping revealed neither directly disrupted known genes nor abnormal expression in EBV-cell lines of selected candidate genes within the vicinity. This absence of plausible candidate genes, which could explain the patients' phenotypes, currently represents a real challenge. There is still the possibility that the observed chromosomal aberrations contribute to at least some of the observed phenotypic abnormalities in these individuals and that only technological limitations restrict our ability to prove this. Therefore, we cannot presently exclude or confirm with certainty the genotype/ phenotype relationship in the studied patients. With future application of systems biology approaches and new technologies, we will be able to further extend our studies.

As submicroscopic copy number variations were found to be causal in a significant proportion of patients with unexplained congenital malformations, in the second part of this project, we analyzed 58 individuals with different types of skeletal anomalies with either real-time quantitative PCR or array CGH.

Based on this approach, we were able to define 10q24 microduplication/ triplication as the molecular cause of Distal Limb Deficiency Micrognathia Syndrome. In addition, we showed that the same chromosomal aberration could also be associated with syndromic forms of SHFM thus extending the clinical spectrum of SHFM3. Our findings not only confirm the existence of DLDMS but also contribute to the understanding of the underlying molecular mechanisms of this congenital limb anomaly. For the first time we demonstrate that the severity of the phenotype is affected by the increased genomic copy numbers within the SHFM3 locus supporting the hypothesis that an altered balance between regulatory elements and their target genes could explain this complex phenotype.

Another original finding was the delineation of two new syndromes within the Cenani-Lenz oligosyndactyly spectrum caused by genomic rearrangements of the *GREMLIN1-FORMIN1* locus- (1) Oligosyndactyly, Radio-Ulnar synostosis, Hearing loss and Renal defects, and (2) Cenani-Lenz-like oligosyndactyly. There are corresponding animal models with a reminiscent phenotype to those of our patients with *GREM1* and *FMN1* aberrations, and future studies should demonstrate to what extent each of these two genes contributes to the observed patients' phenotypes.

Finally, we re-evaluated the clinical phenotype in patients with 2q31 deletions and precisely redefined the genotype/phenotype correlations. We were able to demonstrate that there is no separate SHFM5 locus and that only the *HOXD* genes are involved in the pathogenesis of the observed variable limb defects in individuals with 2q31 deletions. Furthermore, we delineated for the first time the critical loci responsible for the facial gestalt and skeletal abnormalities suggesting the existence of a clinically recognizable 2q31.1 microdeletion syndrome. Future characterization of the deletion size in more individuals with 2q31.1 deletions will allow us to determine to what extent a single gene could be assigned to specific facial characteristics or whether the delineated typical facial gestalt is a result of a continuous gene syndrome.

In conclusion, based on our systematic approach to clinical selection and genetic analysis and by exploiting currently available classical and new techniques, we were able to detect causal genetic abnormalities in 18.5% of the studied individuals. In addition, we found the genetic cause of Distal Limb Deficiency Micrognathia Syndrome, extended the clinical phenotype of the SHFM3 spectrum and delineated three new syndromes- (1) Oligosyndactyly, Radio-Ulnar synostosis, Hearing loss and Renal defects, (2) Cenani-Lenz-like oligosyndactyly and (3) 2q31.1 microdeletion syndrome.

SAMENVATTING

Aangeboren afwijkingen van skelet en ledematen komen voor bij 1/500 à 1/1000 pasgeborenen. De vorming en uitgroei van ledematen is een relatief recente ontwikkeling bij vertebraten. Dit verklaart waarom een groot aantal, reeds bestaande, moleculaire ontwikkelingsmechanismen hierbij werden overgenomen en ingeschakeld. Het is ook de uitleg voor het voorkomen van geassocieerde aangeboren afwijkingen bij meer dan 50 % van deze patiënten, en onderlijnt de noodzaak om deze groep van patiënten multidisciplinair te volgen.

Sinds vele jaren is de ontwikkeling van de ledematen bij vertebraten een belangrijk model geweest voor onderzoek van morfogenese en organogenese in het algemeen, en dit onderzoek heeft geresulteerd in belangrijke nieuwe inzichten.

In dit proefschrift hebben we verschillende klassieke en nieuwe technieken toegepast om een beter inzicht te krijgen in de moleculaire mechanismen van deze groep van aangeboren afwijkingen. Het uiteindelijke doel is een accurate klinische follow-up aan te bieden dankzij een correcte diagnose, die bovendien ook de mogelijkheid biedt voor adequate genetische counseling met de mogelijkheid van eventuele prenatale diagnostiek.

Voor dit onderzoek hebben we een groep patiënten geselecteerd met complexe skeletale afwijkingen na een voorafgaandelijke grondige klinische en morfologische phenotypering.

In het eerste deel van het proefschrift werden 2 patiënten die drager zijn van een zogenaamde gebalanceerde chromosomale herschikking, respectievelijk een translocatie en een inversie, verder moleculair onderzocht. Het betrof een persoon met een mesomelische skeletdysplasie, respectievelijk een persoon met Klippel-Feil syndroom. Onderzoek van de breukpunten betrokken in de chromosomale herschikkingen toonde geen disruptie van bekende genen, noch afwijkende expressie van geselecteerde genen die gelegen zijn in de nabijheid van de breukpunten. Verder onderzoek met nieuwe technologies zal hopelijk meer inzicht bijbrengen in de genotype/phenotype correlatie bij deze twee patiënten.

In het tweede deel van dit proefschrift hebben we onderzoek verricht bij 58 personen met complexe aangeboren skeletafwijkingen met behulp van de zogenaamde microarray-CGH technologie. Als eersten konden we aantonen dat een microduplicatie/triplicatie ter hoogte van chromosoom 10q24 de moleculaire basis is van het zogenaamde “Distal Limb Deficiency-Micrognathia” syndroom. Verder onderzoek

toonde dat deze 10q24 duplicatie/triplicatie eveneens teruggevonden wordt bij patiënten met syndromische vormen van kreefthand-kreeftvoet malformatie (SHFM3). De ernst van de lidmaatafwijking is bovendien gecorreleerd met de toename van het aantal genomische kopijen binnen deze locus.
Een originele bijdrage is eveneens de aflijning van twee nieuwe syndromen binnen het zogenaamde Cenani-Lenz oligosyndactylie spectrum veroorzaakt door genomische herschikkingen in de GREMLIN1-FORMIN1 locus:

1) Een syndroom gekenmerkt door oligosyndactylie, radio-ulnaire synostose, gehoorverlies en nierafwijkingen;
2) Een vorm van oligosyndactylie, gelijkend op Cenani-Lenz syndroom.

Verder onderzoek is nodig om uit te maken in welke mate de GREM-1, respectievelijk FMN1, herschikkingen verantwoordelijk zijn voor deze phenotypes.
In een finaal, derde onderzoeksdeel hebben we het klinisch phenotype bij patiënten met een 2q31 deletie verder gepreciseerd. We hebben aangetoond dat er geen aparte locus voor SHFM5 (kreefthand-kreeftvoet malformatie – syndromische vorm 5) is, en dat enkel de HOXD genen betrokken zijn in de pathogenese van de variabele lidmaat defecten bij deze patiënten.
Verder definieerden we de kritische loci voor het typische faciale voorkomen en de skeletafwijkingen, die resulteren in een klinisch herkenbaar 2q31.1 microdeletie syndroom. Verder onderzoek is nodig om uit te maken of 1 gen of meerdere genen (contigu gen deletie) verantwoordelijk is voor het specifiek faciale phenotype.
In conclusie, bij 18,5 % van de onderzochte patiënten vonden we genetische afwijkingen die causaal zijn voor de skelet/lidmaatafwijkingen. De genetisch moleculaire oorzaak van het zogenaamde “Distal Limb Deficiency-Micrognathia syndroom” werd gevonden, en het klinische phenotype van het “SHFM3 spectrum” werd verder gedefinieerd. Tenslotte werden drie nieuwe syndromen afgelijnd:

1) Het “oligosyndactylie - radio-ulnaire synostose – gehoorverlies – nierafwijkingen” syndroom;
2) een vorm van oligosyndactylie gelijkend op Lenz-Cenani-syndroom en;
3) het 2q31.1 microdeletie syndroom.

1. INTRODUCTION

1.1. The complexities of skeletal biology

The human skeleton (from the Greek word "skeletos", dried up") consists of 206 bones (126 appendicular, 74 axial and 6 ossicles), developing from three distinct embryonic lineages. It serves many key functions including mechanical support for movements, protection of vital organs, and a reservoir of blood and minerals. In total, two tissues (bone and cartilage) and three cell types construct all skeletal elements during the development (chondrocytes and osteoblasts) and postnataly (chondrocytes, osteoblasts and osteoclasts) (Savarirayan and Rimoin, 2002).

The skeleton has to fulfil a series of functions, about which we have little understanding in molecular terms, but which are of critical importance as they are often affected in common degenerative diseases. Among these functions are the molecular mechanisms determining the extent of longitudinal growth, the spatial restriction of extracellular matrix mineralization to bone, and the maintenance of a constant bone mass. These properties raise additional questions about the molecular control of the allocation of specific cell lineages and the determination of the final location and shape of the different skeletal bones.

The remarkably similar distribution of skeletal elements in more than 220 locations in the human and mouse body (to some extent also in the chick) and the significant functional conservation of genes involved in the skeletal development and physiology explain why our ability to generate genetically modified animal models has so dramatically improved the present knowledge about skeletal genetics. Probably there is no other organ in the vertebrate biology that has benefited so much from the study of mouse and chick mutants. In addition, the molecular understanding of the skeletal physiology has increased considerably by analyzing the skeletal cells themselves and their interactions with hormones and the nervous system (Karsenty, 2003; Niswander, 2003).

1.2. Skeletal development (skeletogenesis)

The vertebrate skeleton derives from three distinct embryonic lineages (Figure 1). Undifferentiated descendants of these lineages migrate and/or proliferate to sites in the embryo where skeletal elements will

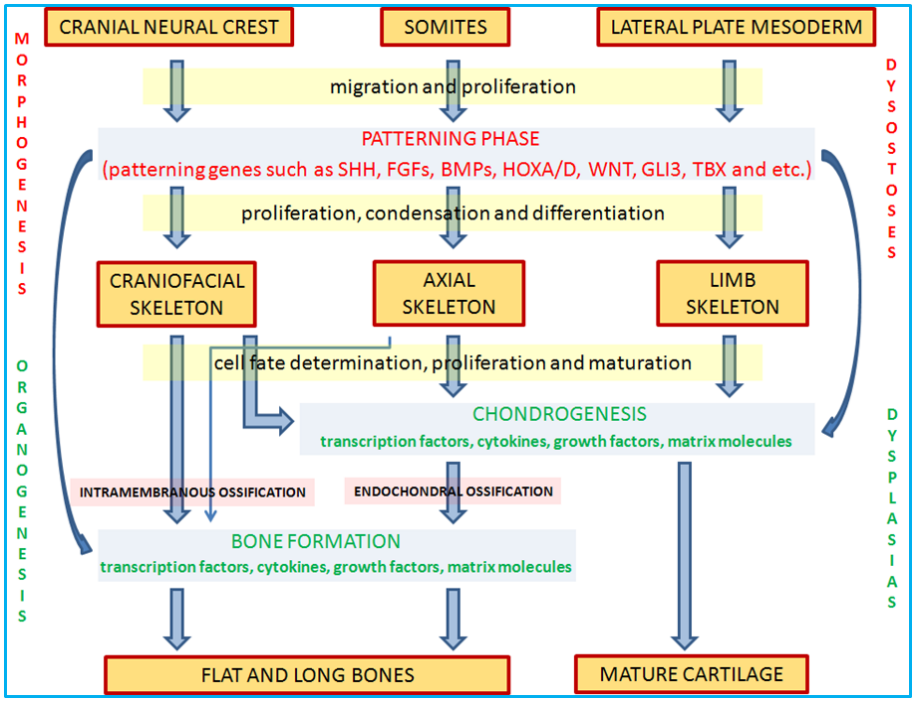

Figure 1. Developmental processes involved in the skeletal patterning and bone formation (modified from Olsen *et al.*, 2000).

develop. The location of the initial skeletal formation will determine which one of these three mesenchymal cell-lineage contributes to particular bones and cartilage of the future skeleton. Cranial neural crest cells migrate from the branchial arches to the craniofacial skeleton, the axial skeleton derives from the paraxial mesoderm (somites), and the limb skeleton is the product of lateral plate mesodermal cells. Later on several morphogenic transformations will occur to form the mature skeleton, called skeletogenesis. In general, the skeletal morphogenesis can be divided into two major phases. During the first phase, following the pattern formation of the general body plan (trunk, head and appendices), characteristic mesenchymal condensations of high cell density will be formed. These condensations outline the pattern of the future bones, referred to as

skeletal patterning. This morphogenesis is under the control of several known major signalling pathways such as Wnts, Hedgehogs, Bmps, Fgfs and Notch/Delta. Precisely adjusted cell fate determination, proliferation and differentiation/ maturation will follow these early patterning events. As a result, a common progenitor cell (osteochondral progenitor) within the mesenchymal condensation will give rise to precursors of both the cartilage (chondrocytes) and bone (osteoblasts) cells. Subsequent vascular invasion will facilitate the last phase of the skeletogenesis, namely the bone formation. In some regions of the craniofacial skeleton and the clavicle, the mesenchymal cell condensations are directly transformed in bone forming osteoblasts (intramembranous ossification). However, in the remaining majority of the skeleton, a chondrocyte differentiation leads to the creation of a framework of cartilage models (anlagen) of future bones, as in the limbs. These anlagen, following the progressing vascularisation, will be replaced by bone and bone marrow (endochondral ossification). Finally, skeletal growth and remodelling after birth will lead to a dynamic adaptation of the skeleton to its functions as a body scaffold providing and supporting movements, protecting internal organs, housing haematopoiesis, and serving as an integral part of the endocrine system. These cascades of pre- and post-patterning events are under a tight genetic control. Often signalling pathways and transcription factors are shared between the two major phases of the skeletogenesis (pattern formation and organogenesis) but in a different context controlling the cell fate determination, proliferation and maturation. Thus, abnormalities of these developmental programs give rise to a cascade of disturbances in the patterning, growth and maintenance of skeletal components and functions. The result is a variety of skeletal anomalies, in particular skeletal dysplasias/dysostoses and arthropathies, that collectively represent a significant proportion of the cases presenting to medical specialists (Olsen *et al*., 2000; Karsenty *et al*., 2009).

Mutations of early patterning genes, which regulate the skeletogenesis, cause disorders called dysostoses (Figure 1). These are most frequently cell-to-cell signalling molecules and transcription factors regulating cell migration, proliferation and fate determination events. The disturbed gene function(s) affect(s) only specific skeletal elements, whilst the rest of the bones may remain unaffected. A primary target of the abrogated morphogenesis could be all three major divisions of the vertebrate skeleton: craniofacial, axial, and appendicular. However, genes/pathways active during the skeletal patterning are also involved in other morphogenic/ organogenic events

so that, when mutated, the resulting skeletal defects are part of (a) syndrome(s) which may include anomalies of non-skeletal tissues. This phenotypic phenomenon is known as pleiotropy and studying different forms of dysostoses has helped to obtain important insights not only into skeletogenesis but also into other developmental processes.

In contrast, mutations in genes that are involved mainly in organogenesis cause disorders called osteochondrodysplasias (Figure 1). In this group, the development and growth of most skeletal elements are affected in a generalized fashion. In addition, the cartilage and bone formation includes the synthesis of specialized extracellular matrices, which also play a crucial role in the developing bones. This is illustrated by a large number of skeletal dysplasias resulting from mutations in matrix molecules (collagen types I, II, IX, X, XI, perlecan, aggrecan) (Olsen *et al*, 2000; Hermanns and Lee, 2001; Yang and Karseny, 2002; Newman and Wallis, 2003; Niswander, 2003; Zelzer and Olsen, 2003; Karsenty *et al*., 2009).

Finally, many genes play an important role in both the skeletal morphogenesis (patterning) and organogenesis (cartilage and bone formation) and as a consequence some inherited bone disorders can display features of both- dysostoses and chondrodysplasias. A good example is Cleidocranial dysplasia (MIM 119600) caused by *RUNX2* mutations.

The present knowledge about the molecular and cellular basis of skeletal development is largely the result of experimental animal studies and investigations of inherited human bone disorders. Transgenic chick/ mouse models and more recently zebrafish mutants helped to explore correlations between specific embryological events and gene functions. This basic knowledge has supported further the research of human disorders with abnormal skeletal development and resulted in the identification of novel genes and pathways involved in skeletogenesis (patterning, chondrogenesis and bone formation), and deepened our understanding of previously characterized molecular mechanisms (Olsen *et al*., 2000; Karsenty *et al*., 2009).

1.2.1. Molecular embryology of the craniofacial skeletal patterning

Neural crest cells from the branchial arches mainly contribute to the craniofacial skeletal patterning under the tight spatio-temporal control of signals between the cells of the neural crest itself and epithelial cells of the surface ectoderm, neural ectoderm or the endoderm. FGF

signalling from the ventral forebrain and pharyngeal endoderm will pattern the pharyngeal skeleton. Later, surface ectoderm FGFs will be required for the frontonasal skeletal pattering. Recent studies demonstrate that BMPs also contribute to the last process (see Rice and Rice, 2008; Yang, 2009).

1.2.2. Molecular embryology of the axial skeleton patterning

The axial skeletal patterning is a result of morphogenetic events leading to segmentation of the paraxial presomitic mesoderm (PSM) and formation of epithelial blocks of segmented mesoderm (somites) on both sides of the neural tube, and the underlying notochord. They form at regular intervals under the control of oscillatory waves of signals in an anterior-to-posterior direction and synchronized left-right symmetry. Definitive patterning of the future vertebrae is formed following a process of re-segmentation and subsequent anterior-posterior fusion of two consecutive somite compartments. The formation of morphological boundaries finally separates these epithelial somites from the PSM.

Soon after their patterning, the somites subdivide into the ventral sclerotome and dorsal dermomyotome, precursors of the future vertebrae, ribs, sternum, and skeletal muscles, respectively. *Shh*, produced in the notochord and the floor plate of the neural tube, determines the sclerotome formation. These sclerotome cells express a high level of *Pax1*. Wnt pathway signals coming from the dorsal neural tube inhibit *Shh,* which in turn inhibits sclerotome formation in the dorsal part of the PSM, and thus induces the development of dermomyotome, which expresses *Pax3*.

Over the last twenty years a series of experimental studies exploring chick, mouse and zebrafish animal models have provided growing information about the molecular content of the oscillatory mechanism controlling the somitogenesis. This process, called the segmentation clock, triggers a cyclic expression of 50 to 100 genes, most of which are components of Notch, canonical Wnt and Fgf signalling pathways. This results in a specific pattern of periodic *Mesp2* expression and thus subsequently defines the ultimate rostro-caudal specification of the future vertebrae.

The Notch signalling pathway mediates short-range cell-to-cell interactions activating the Notch receptor by binding to a ligand (Delta or Jagged) of a neighbouring cell. This process is modulated by glycosylation, e.g. glycotransferases such as Fringe, which can preferentially favour an interaction with Delta. Many cyclic genes like *Hes* family members (*Hes7*), *Lfng*, *Delta* and *Jagged* are downstream

Notch targets. Interestingly, downstream genes in the long-range Wnt/beta-catenin canonical signalling pathway (*Wnt3a*, *Axin2*, *Nkd1* and *Ripply2*) oscillate out of phase with the Notch components. Finally, by creating an opposing rostro-to-caudal PSM expression gradient, *Fgf8*/*Wnt3a* and RA/*Raldh2* define the proper localization of the determination front, the place where the competent PSM cells undergo segmentation. Crosstalk at several levels between all these major players allows a fine tuning of the somitogenesis and a correct axial skeleton patterning.
Mutations in genes involved in the segmentation clock cause an abnormal axial skeletal patterning in animal models. Currently, only five of them have been associated with congenital vertebral malformations in humans - *JAGGED1* (Alagille syndrome, MIM 118450), *NOTCH 2* (Alagille syndrome, MIM 118450), *DLL3* (SCD, MIM 277300), *MESP2* (SCD, MIM 277300) and *LFNG* (SCD, MIM 277300). However, the genetic defects in a few MCA syndromes with vertebral involvement have also been identified, suggesting their involvement in the segmentation of vertebrae: *GDF6* (Klippel-Feil, MIM 148900), *COG1* (CCMS-like, MIM 117650), *HOXD13* (VATER/VACTERL, MIM 192350), *CHD7* (CHARGE syndrome, MIM 214800), *FLNB* (Atelosteogenesis III, MIM 108721; Larsen syndrome, MIM 150250; Spondylocarpotarsal synostosis, MIM 272460), *SOX9* (Campomelic dysplasia, MIM211870), *HLXB9* (Currarino syndrome, MIM 176450), *ACVR1* (Fibrodysplasia ossificans progressiva, MIM 135100), *RECQL4* (Rapadilino syndrome, MIM 266280), *ROR2* (Robinow syndrome, MIM 268310), *NEMO* (Incontinentia pigmenti, MIM 308300), *MKKS* (Kaufman-McKusick syndrome, MIM 236700), *HSPG2* (Silverman syndrome, MIM 224410) and *GPC3* (Simpson-Golabi-Behmel syndrome, MIM 312870). Further research is needed to elucidate the importance of these genes for the axial skeleton patterning (Shifley and Cole, 2007; Dequeant and Pourquie, 2008; Giampietro *et al*., 2009; Yang, 2009).

1.2.3. Molecular embryology of limb patterning

The limb patterning proceeds along three axes during the embryonic development in vertebrates: proximal-distal (PD) starting from the shoulders and ending at the digit tips, anterior-posterior (AP) from the first to the little (fifth) digits, and dorsal-ventral (DV) from the back of the hands/ feet to the palms/ soles. As a result, the three limb segments are formed: (1) the stylopod that contains the humerus and femur, (2) the zeugopod in the middle containing the radius and ulna or tibia and

fibula, and (3) the autopod at the end composed of carpal/ tarsal, metacarpal/ metatarsal bones and fingers/ toes. Any one of these limb compartments has a unique tri-dimensional structure distinguishing it from all the other limb components. This specific pattern is under the tight control of spatio-temporally synchronized and simultaneously orchestrated signals of three important centres derived from the flank mesenchyme early limb primordium called the limb bud: (1) the Apical Ectodermal Ridge (AER), (2) the Zone of Polarizing Activity (ZPA) and (3) the non-AER ectoderm (Mariani and Martin, 2003; Niswander, 2003; Tickle, 2006)

The first morphological indication for limb development is a rapidly growing bulge in the lateral body wall at the positions where future upper and lower limbs will emerge. This early limb bud is composed of proliferating mesenchymal cells from the lateral plate mesoderm covered by ectoderm. The part of the body flank where it appears is called the "limb field". In humans this occurs at days 27 and 28 for the upper and lower limbs, respectively (Nissim and Tabin, 2004). Several data indirectly suggest that *HOX* genes expressed in the intermediate mesoderm probably mark the exact position of the limb field (Stephens and McNulty, 1981; Kessel and Gruss, 1990, 1991; Burke *et al.*, 1995; Rancourt *et al.*, 1995; Cohn *et al.*, 1997)

Soon after the initial budding, an epithelial rim at the distal border of the limb bud is formed, the Apical Ectodermal Ridge (AER). It is covered with overlying periderm and extends from anterior to posterior separating the limb bud into dorsal and ventral parts. Currently, there are no clear data about the molecular mechanisms that initiate the limb bud formation (Fernandez-Teran and Ros, 2008; Zeller *et al.*, 2009). Ffgs and Wnt ligands can induce ectopic limb development (Cohn *et al.*, 1995; Kawakami *et al.*, 2001). A hypothesis suggests that Wnts induce *Fgf10* mesenchymal expression. Later, *Fgf10*, with the support of *Bmp4*, will prompt the AER establishment by the initiation of *Fgf8* expression in prospective AER progenitors and creation of a growth-promoting mesenchymal-ectodermal (m-e) feedback-loop between Fgf8 (produced by the AER-progenitors) and the mesenchymal Fgf10 (Ohuchi *et al.*, 1997; Sekine *et al*., 1999; Agarwal *et al*., 2003; Benazet et al., 2009). The major AER function is to promote the linear PD outgrowth and patterning by keeping the most distal underlying mesenchyme in a proliferative and undifferentiated state. This instructive role for PD pattering is mediated by the production of five Fgfs: *Ffg8*, *Ffg4*, *Ffg2*, *Ffg9* and *Ffg17*. After the AER specification, only *Ffg8* expression is initially observed, equally distributed from anterior to posterior. *Fgf4*, *Fgf9*

and *Fgf17* are later activated in the posterior AER part and subsequently expand in an anterior direction during the progression of the limb development. It was demonstrated that the AER-Fgfs are essential for the limb bud development (Lewandoski *et al*., 2000; Sun et al., 2002; Mariani *et al*., 2008). They are also capable of inducing distal cell identity by activation of *Hoxa11* and *Hoxa13*. This indicates the presence of an Fgf dependent distal fate specification of cells giving rise to the zeugopod and autopod. It was believed that these AER-Fgf morphogenic activities are a result of direct Fgf antagonism with RA signalling from the most proximal limb bud mesechyme (Mercader *et al.*, 2000). As a result, the RA induced *Meis1* and *Meis2* expression remains restricted within the proximal territory of the future stylopod (Captevila *et al.*, 1999; Mercader *et al.*, 2000; Niederreither *et al.*, 2002). However, recent genetic studies suggest that the AER-Fgfs determine the PD limb axis early in the limb development and the specified progenitor pools progressively expand during the later phases of the limb patterning (Dudley et al., 2002; Galloway *et al.*, 2009). In addition, ectodermal Wnt signals interact and support the AER-Fgfs to keep the most distal mesenchyme in an undifferentiated, proliferative state (ten Berge *et al.*, 2008). Beyond the range of these proliferative signals, the mesenchymal cells delay their proliferation, start to express *Sox9* and initiate production of cartilage anlagen of the future bones (Hill *et al*., 2005).

Classical grafting experiments led to the detection of a limb organizer located in the posterior limb bud mesenchyme - the Zone of Polarizing Activity (ZPA) (Hamburger, 1938; Tickle *et al.*, 1975; 1981). Its function is to define the AP limb axis by instructing limb bud mesodermal cells of their final fate depending on to their AP position. The classical Wolpert hypothesis was that it is done by establishment of an AP gradient of a hypothetical ZPA morphogen within the limb bud (Wolpert, 1969). Later studies demonstrated that ZPA derived Shh signalling is both necessary and sufficient to maintain these ZPA functions (Riddle *et al.*, 1993; Chiang *et al.*, 2001; Kraus *et al.*, 2001). The initiation and position of an Shh expressing domain at the posterior limb bud margin is under the control of many transcription factors including *dHand*, *5'-Hox*, *Tbx*, *Alx4*, *Twist*, *Gli3* and *Fgf8* genes as well as RA signalling from the flank (Wang *et al*., 2000; Niederreither *et al.*, 2002; te Welscher *et al*., 2002a; Rallis *et al*., 2005; Capellini *et al.*, 2006; Tarchini *et al.*, 2006; Gonzales *et al.*, 2007; Montavon *et al.*, 2008). In the so-called pre-Shh AP patterning, *Gli3R* and *Alx4* establish the anterior territory by restricting the *dHand* and *5'-Hox* expression to the posterior limb (te Welscher *et al*.,

2002a). This in turn will activate *Shh*. In agreement with this model, it was demonstrated that both dHand and 5'-Hox directly bind to limb specific *Shh* cis-regulatory sequences (Capellini *et al.*, 2006). The *dHand* activity in the posterior region is antagonized by *Twist1* via production of Twist1-dHand heterodimers. Activated posterior Shh signalling inhibits the constitutive processing of Gli3 to its repressor form (Gli3R). The full-length activator Gli3 form acts as transcriptional *Shh* activator. As a result the high Gli3R anterior and high Gli3A posterior expression is consolidated, thus restricting Hand2, 5'-Hox and respectively Shh to the posterior limb bud (Wang *et al*., 2000; Litingtung *et al*., 2002; te Welscher *et al*., 2002b). In addition, Shh establishes an AP concentration gradient by cholesterol promoted diffusion (Li *et al*. 2006). The digit AP identity is therefore patterned by both the spatial gradient and the temporal duration of the Shh morphogenic activity. There is experimental evidence that digit two and part of digit three depend on long-range Shh signalling, whereas the posterior part of the third and the other remaining digits derive from *Shh*-expressing cell descendants. Thus, the last digit is being exposed for the longest time to the Shh activity (Ahn and Joyner, 2004; Harfe *et al*., 2004; Scherz *et al*., 2007). Only digit one does not require Shh signalling. Its development is under the control of other transcription factors such as *Sall4*, *Tbx5* and *Hox* genes (Koshiba-Takeuchi et al., 2006; Montavon *et al.*, 2008). This gradual duration of *Shh* expression most likely provides cells with a temporal memory contributing to the determination of their fate and later digit identification. Therefore this Shh dependant digit specification is mostly linked to the Shh promoted limb bud mesenchyme proliferation (Towers *et al.*, 2008). Interestingly, more recent studies indicate that in fact the Shh promoted digit identities are defined very early in the limb development and Shh is later required for proliferation of the determined progenitors in a temporally uncoupled manner (Zhu *et al.*, 2008). In addition, it is possible that part of the Shh actions are the indirect result of Shh activated downstream targets/signals such as BMPs (*Bmp2*), *Gli1* and *Ptch1*. Currently, despite the absolute Shh requirement for AP patterning of the limbs, there is no direct prove that the Shh gradient also patterns the limb skeleton (Zeller *et al*., 2009).

The crosstalk between the ZPA and the AER was found to be crucial in the complex process of limb formation. Key components of m-e interaction are Shh, limb specific FGFs, BMPs and BMP antagonists like Grem1 (Zuniga *et al*., 1999; Scherz *et al*., 2004). The study of targeted mutations of limb specific *FGF* and *BMP/ BMP* antagonist

genes revealed the existence of self regulatory loops controlling the appropriate initiation and termination of m-e signalling in the coordination of limb development (Khokha *et al*., 2003; Mariani *et al*., 2008; Verheyden and Sun, 2008; Benazet *et al*., 2009). In addition, the correct spatio-temporal expression of these limb morphogens depends on regulatory sequences surrounding the gene landscape in the vicinity (Zuniga *et al*., 2004; Zeller and Zuniga, 2007).

Another aspect of the m-e crosstalk is the establishment of DV limb polarity. An important signalling centre in this process is the non-AER limb ectoderm. Wnt and Bmp signals are key components in the ventralization of both the ectoderm and the mesoderm (Wang *et al.*, 2004). Thus, the dorsal ectoderm expressed *Wnt7a* activates mesodermal *Lmx1b* expression, which in turn is sufficient to pre-pattern dorsal identity of all components of the developing limbs (Parr and McMahon, 1995; Riddle *et al.*, 1995; Vogel *et al*., 1995; Chen *et al*., 1997, Loomis *et al.*, 1998). *En-1* restricts the *Wnt7a* activity to the dorsal ectoderm and thus functions as a ventralizing factor (Loomis *et al.*, 1996; Logan *et al*., 1997). Upstream of *En-1*, Bmp signalling is also required for the early limb bud ventralization. It is mediated by Bmp2, Bmp4, Bmp7, BmpRIA, *Msx1* and *Msx2* to activate the *En-1* ventral expression. There are data that the Bmps may also have an En-1 independent ventralizing effect. Finally, *Wnt7a* in the dorsal ectoderm directly regulates the *Shh* expression within the ZPA (Wang *et al.*, 2004).

The upper and lower limbs share common morphogenic events and morphological regulation during their development. However, there is also an obvious need for functional and corresponding morphogenic difference between them. *Tbx4* and *Tbx5* genes, both T-box transcription factors, were found to be specifically expressed in the lower and upper limbs, respectively (Gibson-Brown *et al*., 1996; 1998). Their expression in the corresponding limb field is present prior to the limb bud initiation (Isaak *et al*., 1998; Logan *et al*., 1998). In addition, there is a synchronized interaction between them, as *Tbx5* down-regulates *Tbx4* in the forelimbs. Conversely, *Pitx1*, another gene specifically expressed only in the lower limbs, up-regulates the *Tbx4* expression levels in the hind limbs. Thus, they seem to be limb specific regulators (Rodriguez-Esteban *et al*., 1999; Takeuchi *et al*., 1999; Logan and Tabin, 1999). Other parallel pathways may also be involved in the control of the upper/ lower limb identities. It is possible that *Hox* genes participate in this process as in general they are important for the AP body patterning (Nissim and Tabin, 2004).

Over the years, a few models were proposed to explain separate aspects of the complex limb development (reviewed by Tabin and Wolpert, 2007; Towers and Tickle, 2009; Zeller *et al*., 2009). However, there is strong evidence for an obviously integrated, three-dimensional function of all currently known pathways in the orchestration of the limb patterning. In an attempt to collate all the available knowledge, Zeller *et al*. (2009) suggested an integrative patterning design for the limb morpho- and organogenesis. During the first phase of limb bud initiation, opposing mesenchymal signals of Gli3, Hand2, 5'-Hox, Bmps, Fgfs and RA pre-pattern the nascent limb bud and support the establishment of the two major signalling centres in the developing limb - the AER and the ZPA. Activation of the AER-Fgf and ZPA-Shh signalling creates morphogen gradients across the limb bud and leads to early specification of the two main limb axes - PD and AP. At this stage cell identities are not yet determined. How the cell fates are specified is not yet known but transcriptional regulators *Hoxa*, *Hoxd* and *Tbx* genes are probably involved. At that time the Bmp activity decreases and the e-m Shh-Grem1-Fgf feedback loop is initiated to promote subsequent proliferation, determination and differentiation. The expansion of the progenitor cell pools is under the control of the self-terminating Shh-Grem1-Fgf signalling, ectodermal Wnts (e-Wnts) and persistent low levels of Bmps. At the time when core mesenchymal cells escape the control of the above signals due to the progressing proliferation, they start to express *Sox9* and undergo chondrogenic differentiation. Proximal to this differentiation front, the PD and AP identities are completely determined thus allowing the initiation of the differentiation process. Therefore, the differentiation of digit patterns occurs the last. At the end of this phase, the Bmp activity again increases as a consequence of Shh-Grem1-Fgf self-termination. The digit identities are later determined with the involvement of *Bmp*, *5'-Hox* and *Sall* genes. No specific regulators of individual digits are currently known (Zeller *et al*., 2009).

1.2.4. Molecular embryology of bone formation

As described above, early patterning mechanisms define the places were future bones will be formed within mesenchymal condensation. Although the intramembranous ossification is different than the endochondral, the participating osteoblasts in these two processes share morphological identity. This suggests common pathways and regulation of the osteochondral progenitors in these two mechanisms of bone formation. Sox9 and Runx2 transcription factors are the first

currently known molecular markers that are required for chondrocyte and osteoblast cell fate determination. The initiation of their expression is coordinated thus that the *Sox9* activity precedes that of *Runx2*. The *Sox9* effects are further enhanced by two other members of the Sox family, *Sox5* and *Sox6* (Lefebvre *et al*., 1998; Akiyama *et al*., 2002). In addition to the *Sox9* and *Runx2* function, Wnt, Ihh, Bmp and Fgf signalling pathways are also incorporated within the process of cartilage and bone formation. Thus, the enhanced Wnt signalling results in an increased bone formation and *Runx2* expression by decreasing the Sox9 levels and the chondrogenic differentiation potential of the bi-potential osteochondral progenitors. Vice versa, a preferential osteoblast differentiation follows the decreased Wnt signalling in both membranous and endochondral ossification.
Ihh activates *Runx2* and osteoblast differentiation only during the endochondral bone formation but does not affect the fate of the mesenchymal progenitor cells. This Ihh function is mediated by two transmembrane proteins, Ptch1 and Smo, which in turn trigger a cascade of events targeting many genes like *Gli1* and *Hip1*. It is not yet clear how the *Runx2* expression is controlled during the intramembranous bone formation. It is possible that the Ihh function there is replaced by Shh.
The Bmp signalling promotes the osteochondral progenitor differentiation to both chondrocytes and osteoblasts via two currently known pathways. The first one (canonical *Smad*-mediated) exploits Bmp type II and I receptors (BmpRII, BmpRIA, BmpRIB and Alk2) to activate Bmp-receptor-regulated Smads (R-Smads) *Smad1*, *Smad2* and *Smad8*. Subsequently these R-Smads bind Smad4 to enter the nucleus and to reach and affect the expression of target genes. In the second (non-canonical) pathway, Bmps activate consecutively *Tak1* and *p38* Mapks. In addition, the Bmp chondro- and osteogenic functions are modulated by inhibitors *Noggin*, *Chording* and *Grem1*.
The importance of the Fgf ligands and their receptors in osteogenesis was elucidated by the discovery that several chondrodysplasias/ dysostoses in humans are caused by mutations in *FGFR1*, *FGFR2* and *FGFR3*. Studying the molecular mechanisms underlying the disturbed skeletal development in patients with achondro-/hypochondroplasia/ thanatophoric dysplasia (MIM 100800/ MIM 146000/ MIM 187600 and MIM 186601) and corresponding animal models revealed that *FGFR3* regulates the chondrocyte proliferation and differentiation. There are no data how Fgfs control the mesenchymal condensation and chondrocyte differentiation. However, there is no doubt about their importance in this process, at least during the intramembranous

bone formation, since *FGFR1*, *FGFR2* and *FGFR3* mutations cause craniosynostoses (Apert syndrome, MIM 101200; Beare-Stevenson cutis gyrate, MIM 123790; Crouzon syndrome, MIM 123500; Pfeiffer syndrome MIM 101600; Jackson-Weiss syndrome MIM 123150; Muenke syndrome MIM 602849; Crouzon-dermo-skeletal syndrome, MIM 134934; Osteoglophonic dysplasia, MIM 166250). Depending on the cell, the Fgfs have a dual function concerning the osteoblast proliferation and differentiation. By activating *Sox2* and thus down-regulating Wnt/ beta-catenin activity, they inhibit the osteoblast differentiation. The opposite effect is via promoting *Bmp2* and suppressing *Noggin* (Olsen *et al*., 2000; Wagner and Karsenty, 2001; Cohen, 2006; Karsenty, 2008; Karsenty *et al*., 2009; Yang, 2009).

1.2.4.1. Chondrogenesis

During the endochondral bone formation, the chondrocytes are the first specific skeletal cell type that appear. They provide multiple functions: (1) being an important player in the process of ossification; (2) allowing and participating in the longitudinal skeletal growth; and (3) forming joints to enable the mobility of the skeleton.
During the early phase of chondrogenesis, mesenchymal osteochondral progenitors within the previously formed mesenchymal condensations start to produce an extracellular matrix (ECM) made of ColI. The Bmps influence this first step of chondrogenesis by controlling the compaction of mesenchymal cells and thus the process leading to cell cohesion in condensations (Barna and Niswander, 2007). Subsequently, at the sites of endochondral bone formation, progenitor cells differentiate into round chondrocytes producing ColII, ColXI and aggrecan under the control of *Sox9* (Lefebvre *et al*., 1997; Bi *et al*., 1999; Akiyama *et al*., 2002; Barna and Niswander, 2007). The aggrecan is a proteoglycan protecting the collagen fibrils and providing mechanical resistance. In addition, together with other proteoglycans it is involved in the Fgf and Shh/ Ihh signalling (Ornitz, 2000; Koziel *et al*., 2004; Settembre *et al*., 2008). The peripheral mesenchymal cells do not differentiate into chondrocytes. They continue to synthesize ColI and form a structure called the perichondrium (Karsenty *et al*., 2009).
Later, the chondrocytes located in the centre of the mesenchymal condensations stop to proliferate and form the prehypertrophic chondrocytes that still produce ColII. These cells express *Fgfr3* to suppress the chondrocyte proliferation via STAT1/ p21 cell cycle inhibition (Peters *et al*., 1992; Colvin *et al*., 1996; Deng *et al*., 1996;

Naski *et al*., 1996; Su *et al*., 1997; Sahni *et al*., 1999). Favourite Fgfr3 chondrocyte ligands are Fgf9 and Fgf18. Fgf18 is produced in the perichondrium under the control of *Runx2* (Liu *et al*., 2002; Ohbayashi *et al*., 2002; Hinoi *et al*., 2006). The function of *Fgf9* is restricted only to the proximal bones (Hung *et al*., 2007).

Ihh is the only hedgehog family member expressed during endochondral bone formation. Its synthesis belongs to the prehypertrophic and early hypertrophic chondrocytes. The Ihh effect to promote chondrocyte proliferation seems to be due to a direct cell action. This process is mediated by the Shh-receptors Ptch1 and Smo as discussed above (St-Jacques *et al*., 1999). Another Shh function is to accelerate the differentiation of the early round proliferating chondrocytes in the growth plates into columns of flat proliferating cells. This is done by two actions that control the column length: (1) a direct stimulation of the cell proliferation at the top of the columns; and (2) an indirect, PTHrP related cell effect of delay of the chondrocyte hypertrophy at the bottom of the columns via the creation of a negative feedback loop with the PTHrP (Vortkamp *et al*., 1996; Kobayashi *et al*., 2005; Koziel *et al*., 2005).

Following the canonical Wnt pathway, Wnt proteins (Wnt1, Wnt3a, Wnt4, Wnt7a, Wnt9a, and Wnt11) inhibit chondrogenesis and chondrocyte proliferation via binding to frizzled and lrp6 receptors (Day *et al*., 2005; Hill *et al*., 2005; Hartmann, 2007). Transcription factors belonging to other families are also involved in the negative (*H1Fα*, *NFatc2*) and positive (*c-Fos*) control of this process (Schipani *et al*., 2001; Wagner and Eferl, 2005).

After exiting the cell cycle, the prehypertrophic chondrocytes progressively become hypertrophic chondrocytes. The last are morphologically well distinct. Stimulated by Mefc2, they produce ColX and express only Fgfr1 (Linsenmayer *et al*., 1991; Karsenty *et al*., 2009). Key regulator of the process of chondrocyte hypertrophy is the *PTHrP* gene. During skeletal development, it is expressed in cells of the perichondrium and the early proliferative chondrocytes. The main chondrogenic PTHrP function is to prevent chondrocyte hypertrophy in an ultimately coupled action with Ihh (Karaplis *et al*., 1994). Wnt and Fgf signals are also involved in the control of this process: Wnt5 and Fgfr3 inhibit, whileWnt4 and Wnt8 accelerate it (Hartmann and Tabin, 2000; Akiyama *et al*., 2004; Murakami *et al*., 2004).

At the same time with the chondrocyte hypertrophy, cells of the perichondrium (bone collar) start to express *Runx2*. One of the *Runx2* targets is to stimulate *Fgf18*. This results in inhibition of further

chondrocyte proliferation and hypertrophy (Ducy *et al*., 1997; Takeda *et al*., 2001; Hinoi *et al*., 2006).
The presence of ColX makes the ECM competent to allow mineralization around the hypertrophic chondrocytes. This also supports vascular invasion from the bone collar under the control of a *VEGF* dependent pathway (Vu *et al*., 1998). The hypertrophic chondrocytes die through apoptosis and are soon replaced by osteoblast progenitors following the invading vessels. These new cells eventually become osteoblasts producing bone ECM with ColI on the top and ColX in the middle. This region, called the primary spongiosa, defines the trabecular bone of the future mature bones.
The ossification process described above occurs centrifugally. The chondrocytes immediately adjacent to the ossification front continue to proliferate. Thus, proliferating and hypertrophic chondrocytes form columns of an avascular structure called the growth plate. This is the place where the continuing process of chondrocyte proliferation, hypertrophy and apoptosis, followed by replacement with osteoblasts, and new-formed bone allows the longitudinal growth of the skeleton (Karsenty and Wagner, 2002; Kronenberg, 2003).

1.2.4.2. Osteoblast and bone formation

Bone formation is the final step of osteogenesis in both endochondral and intramembranous ossification. The coordinated activities of two bone cell types maintain this process - osteoblast and osteoclasts. These two cell lines have different embryonic origin. The osteoblasts derive from the pool of the mesenchymal osteochondral progenitors. They are responsible for the bone formation, for the extracellular matrix mineralization, for the osteoclast differentiation and for the endocrine functions of the bone (Lee *et al*., 2007). The osteoclasts originate from blood myelo-monocytic cells and their main responsibility is the bone resorption and remodelling.
Not surprisingly, due to their common origin, the chondrocytes and osteoblasts share many regulatory mechanisms, e.g. Ihh is, besides its chondrogenic function, also implicated in the osteoblast differentiation of progenitor cells within the bone collar (St-Jacques *et al*., 1999). In addition, Ihh activity is necessary for osteoblast proliferation and survival in postnatal life (Maeda *et al*., 2007). Fgf signalling via Fgf18 and Fgfr2 is the other pathway shared between chondrogenesis and the osteoblast differentiation (Liu *et al*., 2002; Ohbayashi *et al*., 2002). Currently, there are no unequivocal molecular data for the importance of Bmps in this process. However, *Bmp3* inhibits the osteoblast

formation and human *ACVR1* mutations, a gene coding a Bmp receptor type I, cause fibrodysplasia ossificans progressiva presenting with an ectopic bone production (Daluiski *et al*., 2001; Shore *et al*., 2006). These data suggest that in addition to its important functions during the early mesenchymal morphogenic events, this pathway may affect directly the osteoblast differentiation.

LRP5 mutations cause two human diseases associated with an abnormal bone mass, osteoporosis-pseudoglioma syndrome and the high bone mass syndrome. This indicates that Wnt signalling may be implicated in the osteoblast development and control (Gong *et al*., 2001; Boyden *et al*., 2002; Little *et al*., 2002) as LRP5 is believed to be a canonical Wnt co-receptor (Bhanot *et al*., 1996). In the case of osteoporosis pseudoglioma syndrome, a rare form of childhood onset osteoporosis and blindness, loss-of-function mutations abrogate the LRP5 function. In contrast, the high bone mass syndrome is a result of gain-of-function mutations of the same gene. The suggested link between LRP5, canonical Wnt signalling and the osteoblast differentiation was further supported by genetic manipulation of mouse *Lrp5/6* genes or *LRP5* inhibitors as *Dkk1* and *Sclerostin* (Boyden *et al*., 2002; Tian *et al*., 2003; Holmen *et al*., 2004; Li *et al*., 2005; Li *et al*., 2006). However, despite these data, there is not yet direct evidence proving this Wnt dependent LRP5 activity (Karsenty *et al*., 2009). Even more, another hypothesis explains this LRP5 regulatory effect upon the osteoblast differentiation via stimulation of serotonin expression (Yadav *et al*., 2008).

Notch signalling seems to be also directly involved in the developmental control of the osteoblasts. It inhibits the osteoblast differentiation as a result of NICD release and direct NICD-Runx2 interaction. In addition, the osteoblast Notch expression increases the Osteoprotegerin, a TNF factor, and thus suppresses the osteoclast differentiation (Engin *et al*., 2008; Hilton *et al*., 2008).

Three important transcription factors Runx2, Osterix and Atf4 regulate the osteoblast biology at different developmental levels to facilitate the osteoblast differentiation (Ducy *et al*., 1997; Lee *et al*., 1997; Mundlos *et al*., 1997; Nakashima *et al*., 2002; Harding *et al*., 2003; Yang *et al*., 2004). There are also several others that act upstream/ downstream of the first three or independently as inhibitors (Twist1, Twist2) or enhancers (Mef2c, Msx2, Bapx1, Satb2, Nfatc1, Ap1) of this process (El Ghouzzi *et al*., 1997; Howard *et al*., 1997; Karin *et al*., 1997; Tribioli and Lufkin, 1999; Satokata *et al*., 2000; Eferl *et al*., 2004; Kenner *et al*., 2004; Koga *et al*., 2005; Dobreva *et al*., 2006).

1.3. Skeletal dysplasias/ dysostoses

Definition and epidemiology

The skeletal dysplasias are a large, heterogeneous group of genetic conditions characterized by abnormal development, linear growth and maintenance of the human skeleton. There are more than 370 well-recognized forms of osteodysplasias, chondrodysplasias and skeletal dysostoses, which can be delineated by clinical, radiological and morpho-etiological features (Rimoin at al., 2007). Many result in deformity of skeletal cartilage or bone, leading to common features such as short stature, limb mal-alignment, muscle hypotonia and joint contractures. Non-skeletal cartilage may also be involved, resulting in upper airway or respiratory disorders in infancy and hearing defects. Dental, ocular, heart and urinary tract pathology can also be observed.
The overall prevalence rate of congenital anomalies affecting the skeleton reaches almost 16 per 10000 births and congenital skeletal dysplasias represent less than 1/3 of all inborn skeletal abnormalities. The overall frequency of skeletal dysplasias among perinatal deaths is 9.1 per 1000 (Wynne-Davies and Gormley, 1985; Orioli *et al*., 1986; Andersen, 1989; Stoll *et al*., 1989; Rasmussen *et al*., 1996; Dimitrov *et al*., 2000).

Classification and aetiology

The last ISDS Nosology and Classification of Genetic Skeletal Disorders (2006) is still based on an artificial grouping of more than 370 conditions and is built on a combination of morphological, pathogenic/ molecular and/ or pure descriptive criteria. At present, 37 groups of disorders are delineated - 29 osteochondrodysplasia and 8 dysostosis/ congenital limb defect families (Appendix A) (Superti-Furga *et al*., 2007). Within the last 10 years, the rapid progress in molecular genetics and the influence of the Human Genome Project resulted in the elucidation of the underlying genetic defect in more than fifty percent of the skeletal phenotypes included in the last 2006 Classification. Currently disturbed function of more than 160 genes or their regulatory sequences by mutations and/ or genomic deletions/ duplications is the cause of over 260 abnormal skeletal phenotypes. This number of causal single gene and/or genomic aberrations is progressively increasing and resulted in the clinical delineation of new disorders/ syndromes with skeletal involvement, which certainly will find a place in a upcoming revision of the Nosology. On the other hand, despite the fact that the clinically defined entities are presently well identifiable on clinical and radiological grounds, the difference between dysplasias, dysostoses, metabolic bone diseases and

malformation syndromes is becoming blurred. The advent of new technology with the application of systems biology approaches for precise experimental manipulation, fate tracing, conditional gene manipulation, quantitative whole genome screening and computational modelling elucidates the molecular content of basic developmental processes controlling skeletogenesis. It became obvious that conserved developmental pathways are shared at several levels during the morpho- and organogenesis, but in a different context. This logically explains the observed overlapping features of genetic skeletal disorders, which were believed to be the result of different pathogenic mechanisms. Therefore, to promote the understanding of the pathogenesis of individual disorders, parallel classifications have been established. They further subgroup the genetic bone dysplasias/ dysostoses depending on the predominantly affected molecular pathway or based on the disturbed spatio-temporal developmental process during the early skeletal patterning and later osteochondral progenitor differentiation and maturation (Appendix B) (Superti-Furga *et al*., 2001; Kornak and Mundlos, 2003; Newman and Wallis, 2003; Alman, 2008; Phornphutkul and Gruppuso, 2009). Finally, the rapidly growing information in the field requires the development of a unified electronic database which brings together the current knowledge in a user-friendly multidimensional format and serves both the clinic and the research (Rimoin *et al*., 2007).

1.3.1. Acromesomelic/ Mesomelic dysplasias

Dysplasias can result in proportionate or disproportionate short stature depending on the ratio of the trunk-to-limb length. Short limb conditions can be classified according to the site of the shortening as rhizomelic (proximal), mesomelic (middle) and acromelic (distal) dwarfism.

Mesomelic skeletal dysplasias (MSD) (Goldblatt *et al*., 1987) are characterized by limb shortening involving the middle segment (forearm and lower leg). During the last years several distinct disorders of this form of disproportionate short stature have been described. They include Dyschondrosteosis (Leri-Weill), Langer type (homozygous Dyschondrosteosis), MSD Nievergelt type, MSD Kozlowski- Reardon type, MSD Reinhard- Pfeiffer type, MSD Werner type, AD and AR Robinow syndrome, MSD with synostoses, MSD Kantaputra type, MSD Verloes-David-Pfeiffer type, MSD Savarirayan type, AD and AR Omodysplasia (International Nosology and Classification of Constitutional Disorders of Bone 2006-

Appendix C), sporadic cases and a few familial cases of MSD (Ventruto *et al*., 1983; Fryns *et al*., 1988; Brodie *et al*., 1998; Kerner *et al*., 1998; Nishimura*et al*., 1998; Kozlowski and Masel, 1999, Kitoh and Lachman, 2001; Camera and Camera 2003).

1.3.2. Klippel-Feil anomaly (KFA)

The Klippel–Feil anomaly is a clinically and genetically heterogeneous group of disorders characterized by cervical vertebral fusions and may be associated with a variety of other congenital anomalies. The reported incidence varies from 0.02% to 0.71% live births, but most frequently it occurs as an isolated fusion of one or two intervertebral spaces with a prevalence of 7.3/1000 (Clarke *et al*., 1998; Papagrigorakis *et al*., 2003).
Since the original report (Klippel and Feil, 1912), the large number of KFA patients described have shown variable inheritance and a broad spectrum of associated skeletal and non-skeletal congenital defects. This anomaly usually occurs sporadically, but dominant, recessive and X-linked familial forms have also been observed. Several reports suggest autosomal dominant inheritance with reduced penetrance and variable expression, which explains the majority of the sporadically affected individuals. Male and female patients are almost equally presented with a mild, insignificant predisposition to females (1:1.3). Until now, four chromosomal aberration have been associated with KFA: a four-generation familial inversion inv(8)(q22.2q22.3) (Clarke *et al*., 1995), a *de novo* balanced reciprocal translocation t(5;17)(q11.2;q23) (Fukushima *et al*., 1995), a *de novo* pericentric inversion inv(2)(p12q34) (Papagrigorakis *et al*., 2003) and a three-generation familial translocation t(5;8)(q35.1;p21.1) (Goto *et al*., 2006). These chromosomes provided good candidate loci for further research to unravel the molecular mechanisms causing KFA syndrome. Recently, two independent groups were able to identify the first gene implicated in KFA based on positional cloning via translocation breakpoint mapping or candidate gene approach. Affected individuals carried heterozygous mutations in *GDF6* located at chromosome 8q22. Incomplete penetrance for the associated variable ocular defects, vertebral segmentation anomalies and carpal/ tarsal synostoses was noted in both patients and corresponding animal models (Tassabehji *et al*., 2008; Asai-Coakwell *et al*., 2009). Another gene probably involved in KFA is *PAX1*. Analysis for *PAX1* mutations in a group of 63 KFA patients revealed three affected individuals with a *PAX1* sequence variation of significant

physiological impact (McGaughran *et al*., 2003). However, whether it represents the real cause for this entity or a non-pathogenic polymorphism remains unclear.
Several KFA classifications have been proposed (Appendix D) (Giampietro *et al*., 2009). They are based on morphological criteria facilitating the clinical diagnosis, patient management and prognosis. Interestingly, it is difficult to define a single classification subtype of the KFA patients with currently known molecular defects (Tassabehji *et al*., 2008; Asai-Coakwell *et al*., 2009). Furthermore, the phenotype of some of these individuals overlaps with those of more generalized malformation syndromes with vertebral involvement such as Spondylocostal Dysostosis/ Jarcho–Levin syndrome and Wildervanck syndrome. In addition, the genetic aetiology of the majority of the KFA cases remains unknown (Giampietro *et al*., 2009). Therefore, further research should explain the observed pleiotropy and should provide a basis for future phenotype/ genotype correlations, based on a more advanced classification.

1.3.3. Limb Reduction Defects (LRD)

Definition and epidemiology
Limb reduction deficiencies (LRD) are defined as congenital absence or severe hypoplasia of both skeletal and soft tissue limb components. There is high clinical variability of the observed combinations of defects due to the complex architecture of the appendicular skeleton including 120 individual bones, separated in several segments and rays between the upper and lower limbs. With a prevalence rate ranging between 4.5-7.8/ 10000 births, LRDs are the second most common congenital anomaly after the congenital heart defects (CHD). They may present as an isolated malformation (42.1%) or as part of a syndrome (57.9%). The most common associated congenital anomalies (57.9%) are heart (~11.5%), urogenital (~14%) and craniofacial defects (~12.5%). Almost half of the syndromic forms are part of a recognizable disorder (~23%) (Calzolari *et al*., 1990; Froster *et al*., 1992a, 1992b, 1993a, 1993b; Evans *et al*., 1994; Stoll *et al*., 1996; McGuirk *et al*., 2001; Makhoul *et al*., 2003; Stoll- unpublished data).

Classification and aetiology
The currently available clinical LRD classifications are based on description of the missing part of the limb (Swanson et al., 1983; de Smet, 2002; Tonkin, 2006) and they are generally divided into

terminal or intercalary defects and subsequently according to the axis of the deficiency into transverse and longitudinal defects. In addition, based on the missing limb segment they are subdivided in proximal, middle and terminal defects corresponding to the arm, forearm and hand in the upper limb, and to the thigh, leg, and foot in the lower limb, respectively. The deficient bone or longitudinal ray of the middle and distal part of the limb (pre-axial, post-axial, central, radial, ulnar and I-V digit rays) are also often used to further specify the observed anomaly. The idea of these classifications was to unify the terminology across the medical specialities, to provide a basis for grouping patients within a defined nosology, and to promote further research to unravel the underlying aetiology (de Smet, 2002). However, several individual patients fail to fit into any group of the current classifications, thus indicating a need for revision (de Smet, 2002; Elliott et al., 2009).
In an attempt to include only well defined genetic disorders, the last ISDS Nosology and Classification of Genetic Skeletal Disorders 2006 (Appendix E) (Superti-Furga *et al*., 2007) lumps the genetic LRD in one group with 29 separate clinical entities. However, the increasing knowledge over the last years unambiguously demonstrates that the LRDs are inborn errors of development caused by multiple environmental and genetic aetiological factors. In addition, epigenetic modifications and stochastic events substantially contribute to their pathogenesis. Depending on the combination of the complex aetiology and specific spatio-temporal position of disrupted morphogenesis, a variety of abnormal phenotypes occurs, often presenting overlapping clinical features. The observed phenotypic pleiotropy and incomplete penetrance/ expression of the LRDs are probably a consequence of the multifaceted and interlinked developmental mechanisms involved in limb patterning. Further research is needed to delineate how different aetiological components contribute to this process.

Environmental factors causing LRDs
Environmental insults after fertilisation, capable of disturbing the normal morphogenesis and of causing congenital structural defects are coined teratogens, coming from the Greek words “teratos” (monster) and “gen” (creation). However, many teratogenic agents also produce functional disturbances such as mental retardation, impaired vision and hearing loss. Clinical and experimental data demonstrate that several teratogenic factors may preferentially disturb the proper limb development. The resulting limb defects reflect the underling mechanism of abrogated morpho- and organogenesis. Studying these

teratogenic events was thus believed to help our understanding of the complex limb patterning.

There is no other teratogenic agent that has attracted more attention in the society and the medical audience then Thalidomide, prescribed to pregnant women in the fifties of the last century. After being on the market for almost four years its teratogenic potential was suspected and probably more than 5800 infants were identified with a potential exposure (Lenz, 1988). There is a consistent clinical phenotype in affected individuals with distinct bilateral, symmetric preaxial limb reductions with thumb hypo-/ aplasia and/ or more extended radial ray deficiencies. Postaxial/ ulnar ray defects are also observed. The upper limbs are more frequently affected. If there are lower limb anomalies, these involve the femur, tibia and the first toe (Holmes, 2002; Stevenson, 2006; Everman, 2006). Recent experimental studies suggest that its teratogenic effect is due to primary blocked angiogenic outgrowth during early limb development and/ or induced Caspase-dependent cell death (Knobloch *et al*., 2008; Therapontos *et al*., 2009).

A well-documented teratogenic drug with skeletal effect is the anticonvulsant Phenytoin. Affected individuals have hypoplasia of distal phalanges, shortness of metacarpals, cone-shaped epiphyses, limited movements at interphalangeal joints and tapering fingers with nail hypoplasia. These skeletal anomalies are often observed only after an X-ray examination (Lu *et al*., 2000; Holmes *et al*., 2001; Holmes, 2002). Terminal transverse limb reductions are infrequently reported and suggest a vascular disruption to explain at least partially the Phenytoin teratogenesis (Sabry and Farag, 1996). Similar terminal transverse LRDs affecting distal phalanges and tubular hand bones are also observed after intrauterine exposure to the anticoagulant Warfarin and the anticonvulsant Valproic Acid (VA). However, thumb hypoplasia/aplasia and other preaxial/radial ray deficiencies are the most commonly observed limb malformations in the VA embryopathy (Holmes, 2002).

Variable transverse reduction defects are induced by Misoprostol, a synthetic analog of prostaglandin E_1. This drug was often prescribed for initiation of illegal abortions in Latin America. Its pathogenic effect is due to stimulation of uterine contractions resulting in hypovolemia and hypoperfusion of the embryo or the foetus and terminal vascular disruption limb anomalies as a consequence (Los *et al*., 1999; Coelho *et al*., 2000).

There are additional environmental factors that cause RLDs due to vascular compromises. Examples are maternally related conditions

such as amniotic bands, other constrictive forces (uterus malformations, tumours) (Stevenson, 2006; Everman, 2006), thrombophilia (Hunter, 2000) and thalassemia (Lam *et al*., 1997; Li and Li, 2008) or maternal addictions such as cocaine use during the pregnancy. It is believed that they disrupt the limb morphogenesis via vascular insults. Other causes are mechanical forces producing trauma during pregnancy like chorionic villus sampling (Firth *et al*., 1991; Stoler *et al*., 1999; Holmes, 2002), cervical dilatation, curettage, placental trauma, amniocentesis (Lamb, 1975; Hall, 1996; Viljoen, 1995; Holmes, 1995, 1997) and selective reduction of multiple gestations (Holmes, 2002; Simeonov and Dimitrov- unpublished data).

Diabetes mellitus is another maternal condition with an indisputable teratogenic effect. Children born to diabetic mothers have a two- to eight-fold higher risk of congenital anomalies than the general population. The heart, CNS, spine and limbs are the most commonly involved. Almost a third of the infants with malformations present with limb anomalies (Holmes, 2002; Stevenson, 2006; Everman, 2006; Frias *et al*., 2007). The lower limbs are predominantly affected often in combination with abnormalities of the lower spine. A specific phenotypic pattern with caudal deficiency, preaxial hallucal polydactyly and femoral hypoplasia/unusual faces syndrome (MIM 134780) is 65-200 times more frequent in these patients, challenging the correct differentiation between a teratogenic effect versus a molecular defect to explain the phenotype (Farrell *et al*., 2002; Yang *et al*., 2006; Frias *et al*., 2007; Correa *et al*., 2008; Adam *et al*., 2009). In all individuals with skeletal involvement there are associated defects of the heart (84.6%), kidney (50%), ears (38.8%) and cleft palate (33%). The combination of anomalies may overlap with the VACTERL association and OAVS (Wang *et al*., 2002; Frias *et al*., 2007; Castori *et al*., 2008). Hyperglycemia and subsequent fetal oxidative stress with high levels of free reactive radicals are supposed to be the primary teratogenic mechanism (Allen *et al*., 2007; Eriksson, 2009). However, this general pathway cannot explain sufficiently the predilection to specific patterns of congenital anomalies. The diabetic predisposition itself does not seem to be directly related to this. Epidemiological human and molecular animal studies suggest the presence of a multifactorial effect of oligo-/polygenic susceptibility and environmental triggers which determine the presence of particular disruption during the embryonic development. This complex oligo-/polygenic and multifactorial pathogenic mechanism may be applied not only to the diabetic embryopathy, but also to other teratogenic

drugs/ chemicals (Pavlinkova *et al*., 2008; Eriksson, 2009; Zhu *et al*., 2009).
LRDs are also sporadically observed after intrauterine exposure to several other teratogens such as ethanol, aminopterin (methotrexate), retinoic acid, fluconazole, smoking cigarettes, varicella and hyperthermia (Holmes, 2002; Stevenson, 2006; Everman, 2006).

Genetic factors causing LRDs
Several inheritable AD, AR and X-linked forms of isolated and syndromic LRDs are described (Superti-Furga *et al*., 2007). In addition, chromosomal aberrations (6.8% of all RLDs) are also consistently associated with congenital limb deficiencies. A small proportion of patients with trisomy 18 (less than 10%) can have absent radial/ tibial ray and oligodactyly (Christianson *et al*., 1984; Stevenson, 2006; Everman, 2006). Thumb a(hypo)plasia and other distal limb anomalies are associated with terminal deletions of chromosome 13q (13q31.3–q33.1 and 13q33.3–q34) (Kirchhoff *et al*., 2009). Severely a(hypo)plastic distal phalanges are characteristic for the 9p duplication syndrome (Fryns *et al*., 1979; Zou *et al*., 2009). Radial ray reductions and other skeletal defects are rarely reported in patients with 22q11 deletion syndrome (Digilio *et al*., 1997; Kokitsu-Nakata *et al*., 2008; Simeonov and Dimitrov- unpublished data). Severe reduction limb anomalies/ monodactylous ectrodactyly have been observed in a few individuals with 2q31 deletions suggesting a new SHFM5 locus (Del Campo *et al*., 1999; Goodman *et al*., 2002; Bijlsma *et al*., 2005). Based on phenotype/ karyotype correlations of published patients with SHFMs and associated chromosomal aberrations, Niedrist *et al*. (2009) proposed two additional loci - chromosomal bands 4q32–q35 and 6q16–q22 - that are significantly associated with this phenotype. Several additional chromosomal abnormalities can also result in LRDs such as trisomy 13 (radial aplasia), triploidy (radial aplasia, oligodactyly), Turner syndrome (45, X), Klinefelter syndrome (47, XXY) and trisomy 21.
The Human Genome Project with the introduction of new techniques for whole genome analysis and the establishment of worldwide collaborative databases collecting data on patients and/ or animal models significantly improved our knowledge about the molecular basis of many genetic disorders, including the genetics of monogenic forms of LRDs. In addition to well known disorders like Holt-Oram syndrome (*TBX5*), Ulnar-mammary syndrome (*TBX3*), the EEC/ EEM/ ADULT/ SHFM4 phenotypic spectrum (*p63*), Acheiropodia (*LMBR1*), Okihiro syndrome (*SALL4*), Cornelia de Lange syndrome

(*NIPBL*) and Roberts syndrome (*ESCO2*), many new phenotypes were delineated and the corresponding molecular defect identified. Thanks to the application of array CGH technique in the analysis of patients with unknown diagnosis, new chromosomal deletions/ duplications causing LRDs were found with the discovery of new monogenic (*FAM58A* and STAR syndrome- Unger *et al*., 2008) or genomic disorders (1q21.1 microdeletion in TAR syndrome - Klopocki *et al*., 2007). In addition, the difference between single gene defects and genomic syndromes became blurred since the newly detected genomic rearrangements may be Mendelian inherited (de Mollerat *et al*., 2003, Klopocki *et al*., 2007).

In the last five years several new genes have been implicated as a cause of different inherited/ inborn syndromes in individuals with an abnormal skeletal development, particularly the group of the oligodactylies and SHFMs. Oligodactyly is the congenital absence of digits. More than one digital ray may be involved. The number of affected limbs and the symmetry of the observed defect may vary. Depending on the AP polarity the deficiency can involve only anterior, posterior, central ray digits or all of them. In some patients the severity of the defect makes the recognition of specific digit patterns impossible. All bones of the hands and feet may be involved and syndactyly between the remaining digits is often observed. In this clinical spectrum of terminal limb defects, SHFM (ectrodactyly) is defined as an absence of central digit rays. However, clinical, experimental and molecular data suggest that there is a high clinical variability starting from the classical bidactylous SHFM phenotype, through different levels of missing digits to a total lack of hands and feet. In addition, SHFMs may be part of a syndrome or an isolated anomaly. Arbitrary, this continuum of abnormalities was divided in classical and atypical SHFM forms based on the type of limb defects present.

Until recently, five SHFM loci were defined on clinical findings and genetic mapping: SHFM1 at 7q21.2q22.1; SHFM2 at Xq26; SHFM3 at 10q24q25; SHFM4 at 3q27 (*p63*); and SHFM5 at 2q31. The *p63* gene causes SHFM4 (Basel *et al*., 2006). There is variable expressivity amongst the *p63* mutations (Rinne *et al*., 2006, 2007). For the SHFM3 locus, the limb defects in humans are associated with a unique, most commonly 500Kb 10q24.31q24.32 microduplication (de Mollerat *et al*., 2003). Based on analogy with the corresponding *dactylaplasia* (*dl*) mouse model, *FBXW4* was suggested as the most plausible candidate gene responsible for the phenotype. Later studies disagree with this hypothesis and favour a disturbed balance between

regulatory sequences and causal genes in the chromosomal rearrangements (Kano *et al*., 2007; Friedli *et al*., 2008; Dimitrov *et al*., 2010).

New SHFM loci were reported: SHFLD6 at 1q42.2-q43 and 6q14.1 (Naveed *et al*., 2007); SHFLD7 at 2q14.2 (Babbs *et al*., 2007); SHFLD8 at 17p13.1–p13.3 (Lezirovitz *et al*., 2008); and SHFM9 at 12q13.11–q13 (*WNT10b*) (Ugur and Tolun, 2008). Two additional loci, at chromosomes 4q32q35 and 6q16q22 (Niedrist *et al*., 2009), provide promising options for further research. In addition, the phenotypic spectra of SHFM1 and SHFM3 were extended. A 7q23.1 microdeletion removing only three genes, *DLX5*, *DLX6* and *DSS1*, was found in patients with Fontaine syndrome (Bigo *et al*., 2009). Finally, DLDMS and syndromic forms of SHFM were associated with 10q24 microduplications and linked to the SHFM3 locus (Dimitrov *et al*., 2010). Therefore, a future LRD classification should certainly reflect these new data.

1.4. How rare are the congenital limb defects and is it worth to study them?

Since many years the vertebral limb development serves as an important model to show how different processes and pathways are synchronized and involved in morpho- and organo-genesis. Because of relatively easy accessibility and experimental manipulation at an acceptable cost level, the chick limb patterning was the pioneer model used to study fundamental developmental processes in the second half of the last century. As limb formation is not necessary for embryonic survival, several spontaneous and genetically manipulated (transgenic) animal models contributed to the discovery of important signalling pathways and their molecular content involved in limb and skeletal development. As several of these key genetic players have an important role in other developmental cascades, this knowledge contributed significantly to the understanding of pattern formation of other tissues and organs (Niswander, 2003; Tickle, 2006). However, despite this substantial contribution to developmental biology and genetics over the last five decades, the genetic studies of LRDs in man have been a relatively neglected topic. At present there are only a few genes that cause such phenotypes compared to other genetic skeletal disorders, e.g. osteochondrodysplasias. This is mainly due to some widely accepted misleading prejudices. The first is the assumption that they are very rare and not life-threatening. The second is that there is no curative treatment possible. Even if the molecular defect is known there will not be a fast and easily visible benefit to the society because it will not lead to the immediate development of a new treatment strategy different from those currently available (e.g. the surgical correction). The automatic consequence is poor research interest within the medical specialities dealing with these patients, namely surgery and clinical genetics, as the chance of a successful grant application is negligible. This is unfortunate, not only because the contribution limb malformation studies would make to human health and developmental biology, but also the great impact and potential knowledge this would bring to developing branches in medicine and the biomedical industry.

Like most genetic diseases, limb anomalies are individually rare but taken together, congenital skeletal defects occur in 1 in 500 to 1 in 1000 births (Wilkie, 2003; Ferretti and Tickle, 2006). Because of this elevated frequency and severe, long time burden they are of significant clinical and social importance. In addition, the limb formation was a relatively recent event in the evolution of vertebrates and many developmental pathways controlling the limb/ skeletal

development were co-opted from already existing patterning programs of other body parts. Therefore, LRDs are often (~58%) associated with other congenital defects. Based on this pleiotropy, genetic studies of diseases that affect the skeletal development and growth provide invaluable insights about the role of not only individual gene(s), but of entire developmental pathways into the morphogenesis of many non-skeletal organs (e.g. Holt-Oram syndrome, *TBX5* gene and the heart development). Different mutations in the same gene may result in different phenotypes, and disease "families" are frequently caused by mutations in different genes as components of the same pathway. The correlation of these clinical phenotypes with the identified allelic changes provides a sensitive method for the analysis of structure-function relationship and elucidates the intimate molecular pathophysiology of the observed dysmorphogenesis. Phenomena such as poly-A extension tract mutations (*HOXD13* and *HOXA13*), gain-of-function mutations (FGFRs), mutation hot-spots (*FGFR2*) and sperm cell mutation selective advantage (*FGFR2* and *FGFR3*) have been discovered as a result of limb defect studies. Other observations, e.g. the sex-limited segregation distortion in SHFM with excess of affected male offspring born to affected fathers, remain to be explained (Jarvik *et al*., 1994; Wilkie, 2003).

The analysis of human limb anomalies provides important information that complements data from experimental surveys of gene function in animals and cells, as even the closest evolutionary mouse models are not always sufficient to study and to discover causal genes for human skeletal disorders due to interspecies differences in development (this includes both qualitative and quantitative dissimilarities). Dominant phenotypes in man may only be observed in homozygous mice or in certain mouse strains. In addition, the study of the phenotypic consequences of gene mutations in animal strains demonstrates the gene function under conditions of minimal environmental and genomic modifications. In contrast, familial and human population studies can elucidate the plasticity and adaptability of the genome under the pressure of a variable environment. The abnormal phenotype may result from a combined effect of genetic and environmental factors, but can also be due only to an environmental insult. Therefore a correct diagnosis may be difficult, but important with significant impact on correct genetic counselling. It is often believed that terminal transverse, asymmetrical and/or single LRDs suggest a non-genetic aetiology and in general a low familial recurrence risk. However, clinical data demonstrate that this is not unequivocal. LRDs escaping clear Mendelian inheritance may be

caused by single gene disorders via a disruptive mechanism. This is the case in foetuses with homozygous *HBA1*/ *HBA2* mutations and terminal transverse limb abnormalities or in patients with Fanconi anaemia and asymmetric limb deficiencies. In both situations the observed LRDs are a consequence of the chronic foetal hypoxia (Lam *et al*., 1997; Joenje and Patel, 2001; Wilkie, 2003). A non-Mendelian inheritance pattern of single gene phenotypes can also be the consequence of titrating effects of multiple alleles or loci (oligogenic/polygenic), gene pleiotropy, environmental and/ or stochastic events (multifactorial). For example, the variable expression and penetrance of SHFLD6 is probably due to biallelic inheritance (Naveed *et al*., 2006). A complex, non-Mendelian inheritance pattern as a result of single gene mutation was found in individuals with variable skeletal and associated non-skeletal anomalies carrying *GDF6* or *PITX1* mutations. The differences of the allele size effect upon the inconsistent patient phenotype were explained with the inconstant degree of redundancy and/ or variability of the pleiotropic *GDF6* or *PITX1* thresholds required for normal skeletal/ non-skeletal development among the affected individuals (Gurnett *et al*., 2008; Tassabehji *et al*., 2008; Asia Coackwell *et al*., 2009). Finally, studying teratogen induced limb defects, particularly Thalidomide, substantially changed the modern pharmacogenetics and drug marketing (Wilkie, 2003).

Nowadays, the impact of the information gathered by the analysis of abnormal human limb development is enormous and it gradually changed the scientific approach and study of complex developmental processes. In addition, it increased our understanding how disorders evolve and progress. Modern methodologies benefit from molecular genetic studies of inherited skeletal syndromes to study common population diseases such as osteoarthritis, rheumatoid arthritis and other degenerative skeletal disorders (Ikegawa, 2006; Kannu *et al*., 2009). This knowledge substantially contributed to the development of new therapeutic approaches in medicine, e.g. the regenerative cartilage and bone medicine. One cannot imagine application of BMPs, FGFs and mesenchymal stem cells for bone/ cartilage healing, repair of bone defects or treatment of osteoporosis without the available basic information about limb and skeletal development (Beenken and Mohammadi, 2009; Chen and Tuan, 2008; Karsenty *et al*., 2009; Richardson *et al*., 2009; Sensebe *et al*., 2009; Yang, 2009).

Last but not least, the opportunity to provide a correct diagnosis is of great importance for the individual patients and their families. The ability to provide a reliable prognosis and the possibility of prenatal

diagnosis depends on knowing of the molecular cause (Scambler, 2006). In addition, the establishment of a systematic approach for assessment, management and genetic analysis of patients with LRDs will certainly lead to a chain-reaction of discoveries of new causal genes at a reasonable cost, disproving the prejudice that they are very rare and not important. Finally, from the point of view of the presently proposed personalized and preventive medicine, this will gradually improve the access to welfare and social support of the affected individuals and their families (Downing, 2009; Ginsburg and Willard, 2009; Tweardy and Belmont, 2009; Collins, 2010). Of course, a multidisciplinary approach and open communication between specialists of different branches in bio-medical science are essential factors in this process.

2. AIMS OF THE PROJECT

The congenital skeletal and limb defects are a heterogeneous group of malformation syndromes, most of them with unknown genetic cause. There are several classical position-independent and positional strategies for cloning of new disease genes such as using previous knowledge about the protein product, through an animal model, linkage analysis or genome wide association studies. However, for sporadic and rare genetic disorders these approaches based on positional cloning, physical mapping or candidate-gene sequencing may be inefficient. For such conditions, mostly severe or lethal phenotypes, investigating patients who are carriers of chromosomal abnormalities such as translocations, inversions or micro-deletions/ duplications may help to identify their genetic cause.

In this project two patients, a girl with a mesomelic form of skeletal dysplasia, congenital glaucoma and *de novo* translocation (13; 18)(q14;q23), and a second non-related female with Klippel-Feil syndrome and *de novo* inversion (2)(p12q34), were selected for further genetic analysis. In addition, a selected group of patients with (1) complex skeletal phenotypes, combining features of two Mendelian conditions, (2) with a Mendelian condition plus mental retardation or (3) with an unknown molecular defect were analysed for the presence of genomic micro-deletions/ duplications. The aims of the study were to identify the molecular aetiology of these rare skeletal malformation syndromes and to increase the current knowledge about the underlying molecular mechanisms involved in skeletal and limb development. Therefore, classical and new techniques for positional cloning and candidate gene(s) selection/ validation were applied in a "positional candidate gene" approach.

Finally, with the detection of the genetic cause of the selected rare skeletal phenotypes, we aimed to improve the clinical management and genetic counselling of individuals with congenital skeletal malformation syndromes and to contribute to the development of an advanced classification of this group of genetic skeletal disorders.

3. MATERIALS AND METHODS

Patient's material

Sixty-five patients were selected for further genetic analysis.

- two carriers of apparently balanced chromosomal translocations/ rearrangements and associated skeletal abnormalities, KFA and MSD;
- forty-two patients with unexplained syndromic forms of skeletal anomalies for array CGH screening;
- five patients with a 2q31.1 deletion for detection of the deletion size by array CGH;
- sixteen patients with Holt-Oram syndrome for intragenic deletion/duplication screening;

In all patients conventional GTG-banded cytogenetic analysis at a resolution between 650 and 850 bands had been performed previously.

Patients' DNA extraction

Peripheral blood cell DNA from patients, parents and analysed relatives was obtained, following standard protocols. The quality and yield of extracted DNA was checked by NanoDrop ND-1000spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

Fluorescence in situ Hybridization analysis (FISH)

FISH analysis was used for: (1) fine breakpoint mapping by "walking FISH"; (2) primary screening for CNVs; (3) confirmation of array CGH data; and (4) analysis of the genomic architecture of detected CNVs. The probes were constructed with two alternative methods. In the first way, 1μg genomic DNA isolated from BACs and fosmids with the Nucleobond AX100 kit (Machery-Nagel, Dtiren, Germany) was labelled using incorporation of either biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) by nick translation system (Invitrogen, Carlsbad, California, USA) or digoxygenin by DIG Nick mix (Roche Diagnostics, Mannheim, Germany). The BAC and fosmid clones were selected exploiting web-based electronic sources (Ensembl and UCSC Genome Browsers) and requested from the BACPAC (BPRC) Resource Centre at the Children's Hospital Oakland Research Institute (BPRC-CHORI, Oakland California, USA) (Krzywinski *et al*., 2004). Alternatively, DOP amplified BAC DNA used for construction of array CGH platforms was labelled in SpectrumOrange™- dUTP (red signal) and SpectrumGreen™- dUTP (green signal) (Vysis, Abbot laboratories, Chicago, Illinois, USA) by a

second DOP amplification or random labelling (Invitrogen, Carlsbad, California, USA) (Backx *et al*., 2008). The hybridizations were done on metaphase chromosome spreads or interphase nuclei of peripheral white blood cells, EBV-immortalized lymphoblasts and skin fibroblasts as described (Menten *et al*., 2006, Backx *et al*., 2008). The signals were visualised by digital imaging microscopy with CytoVision capturing software (Applied Imaging, Santa Clara, California, USA).

RNA extraction and cDNA synthesis

Fibroblasts and EBV-immortalized lymphoblasts from the patients and control individuals were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) at 37°C under 5%CO_2. Total RNA of the cultured cell lines was extracted with the RNeasy Kit (QIAGENE, Venlo, Netherlands) according to the manufacturer's protocol. The samples were treated with DNaseI (Roche Applied Science, Vilvoorde, Belgium) to avoid DNA contamination. The quality and yield of extracted RNA were checked by NanoDrop spectrophotometer (ND-1000). Subsequent cDNA synthesis was performed using mixture of equimolar proportions (1:1) of poly-A and random primers (Invitrogen, Carlsbad, California, USA and Amersham, Diegem, Belgium) and Superscript III RNase (Invitrogen, Carlsbad, California, USA) according the manufacturer's protocol.

RT Q-PCR analysis of genomic DNA

SYBR Green assays were designed using the program PrimerExpress v2.0 (Applied Biosystems, Foster City, California, USA) with default parameters in every case. To ensure they were unique for the region under study, the amplicon sequence and primer specificity were checked by BLAT and UCSC *in-silico* PCR (Kent, 2002; Karolchik *et al*., 2003). The efficiency of each assay was tested using serial dilutions of control DNA. Primer sequences and locations of the assays are provided in Table 1. All reactions used 10ng of genomic DNA and qPCR mastermix (Eurogentec, Seraing, Belgium). PCRs were set up in a 15ml volume in 96-well plates with two replicates per sample. Reactions were run in an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, California, USA) with standard conditions. Melting curves were implemented during the reactions to check for the possibility of primer-dimer formation. For data analysis, Ct values were obtained using SDS 2.0 (Applied Biosystems, Foster City, California, USA). Input DNA quantities were normalized to one assay from the *p53* gene locus. Relative DNA copy

numbers were obtained by pair-wise comparisons of test and control DNAs using ΔΔCt method (Livak and Schmittgen, 2001). Each assay of interest was repeated two times, thus providing in total four runs per sample. Means of these two independent experiments (four runs), with standard deviation (SD) less than 0.15, were used for final fold difference calculation. The computational analysis was carried out in Excel 2003 (Microsoft Corporation, Redmond, Washington, USA).

Table 1. Primers for RT Q-PCR analysis of genomic DNA

No	ID	Forward primer 5'→3'	Reverse primer 5'→3'
		***TBX5* gene**	
1	*TBX5ex1*	GGAAGAAGACCTGGCCTAAAGAG	GCAGGGCTCAGGTGAAGACT
2	*TBX5ex2*	GCCTTCACCCAGCAGGTAAG	AAAGCCAGACTCTGACTTTGATCTC
3	*TBX5ex3*	CCACTACTGCCAGGGCTTGT	GAGAAACACTTTGATTCCCTCCAT
4	*TBX5ex4*	CGTTTCTCCAGGAATTGCTTTT	GCGGGAATTAATGCCAGTATTT
5	*TBX5ex5*	CGGTGCAGTGCGCTACCT	CCGTCACAGACCTAGATGAAGGA
6	*TBX5ex6*	CCAGCCTAGATTACACATCGTGAA	GTGAGTGCAGAACGCTGTATTTTT
7	*TBX5ex7*	CCAGGTAAGCCAGCAGCAA	ATTTCCATGTGCCTGGCATT
8	*TBX5ex8*	GCCTCCACCCAACCCATAC	CCTTTCCTCTTGGTACAATGGTAAA
9	*TBX5ex9*	GACGCTCCCTGGCATCCTA	GCGACAGACAGCTGGAAAGAC
		Chromosome 10q24 (SHFM3) locus	
10	*BTRCintr2 (Pr1)*	CTGCCAACCAGATTGGAATTC	TCTTAATCCTGTTTGGAGCTGTCA
11	*FGF8ex6 (Pr2)*	GGTGTCTGGCTCCCCTACCT	CTCCAGCACAATCTCCGTGAA
		Chromosome 15q13.3 (*GREM1-FMN1*) locus	
12	SNP_A-4240484	AAGAGCCAGCATGGCTGTAAG	TCAGCAGCTTTTAATGCAAGTTTG
13	SNP_A-4201883	GAAGGTGTTGCAGGACAAACC	TGACCAGCTCTTCTCTGTTCATTG
14	SNP_A-2241478	TGTGAGGCTCACAGCACTCTAAG	CCCCTTTGGATGTGTTCCAA
15	SNP_A-2176534	GGGTGGAAAGATCAAGGAAACAC	AGACTGTTGCCCATCCATGAG
16	SNP_A-2233558	AGAATCAGCTAGTCTTTCTCAGAACTCA	ACAGGAGCCAGTTGCACACA
17	SNP_A-1865339	TCAAACTCAGGTACTGCATGCTTT	TTTTCCTTTGACACTTCAGCAACT
18	SNP_A-1854043	GGCCTGTTCTAGGCTTTCCA	AATGAACAGTAACTACAATATGTGGTATTCC
19	SNP_A-1949971	ATCTTATCCCCTTCCCTTTCTATTG	CAACAGAGCCCTTGGTATGGA
20	SNP_A-2022976	GCTGTGAGAAGTGTCTTCGTGTTATT	CTCGCCTCACTCACAGAAGCT
21	SNP_A-2284222	TGCTCCAGGCAGGTCAGATC	GGCTGATGGTCATTCACACAGT
22	SNP_A-2248182	AATCTGTGAAAGCTCAAGGGAAA	GTTGGAAGCTTTGAGAGCAGAAG
23	SNP_A-2124352	GGTGATTTGCCCTCGTTTTC	AACTTCCCATGCCACAGCTT
		Chromosome 17 (p53) locus	
	p53	CCCAAGCAATGGATGATTTGA	GAGCTTCATCTGGACCTGGGT

RT Q-PCR analysis of RNA

Quantitative transcript analyses of *ORMDL1*, *GDF8*, *LRCH*, *SUCLA2*, *VDRIP*, *DIRC1*, *GULP*, *PMS1*, *ITM2B*, *LRRTM*4, *HsG10342, SALL3*, *Esterase D*, *WDR75*, *GREM1* and *FMN1* were performed. The RNA real-time quantitative PCR was carried out using SYBR Green assays. Intraexonic or exon-exon-boundary-spanning primers were designed by PrimerExpress v2.0 (Applied Biosystems, Foster City, California, USA) software with default parameters (Table 2). Before the cDNA conversion, RNA aliquots were taken and used later on as a non-amplification control to check the quality of the DNaseI treatment and DNA contamination. The amplification efficiency of each assay was checked by a cDNA dilution series (Livak and Schmittgen, 2001). For all reactions 50ng cDNA and qPCR mastermix (Eurogentec, Seraing, Belgium) in a 15µl volume in duplicates were used. Reactions were run in an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, California, USA) with standard conditions. To calculate relative expression levels Ct values (SDS2.0 Applied Biosystems, Foster City, California, USA) were normalized in a two step procedure according the ΔΔCt method (Livak and Schmittgen, 2001). Combination of means of seven reference genes Cts, *ACTB*, UBC, *GUSB*, *B2M*, *CLK2*, *GAPDH* and *YWHAZ*, was used for the first normalization (Vandesompele *et al*., 2002). Subsequently, each individual patient's value was normalized to the mean value of at least three control individuals. All assays of interest were repeated two times, thus providing in total four runs per sample. Means of these two independent experiments (four runs), with standard deviation (SD) less than 0.15, were used for final fold difference calculation. Calculations were carried out in Excel 2003 (Microsoft Corporation, Redmond, Washington, USA).

Array CGH analysis

Four different array CGH platforms were used for: (1) primary screening for CNVs; (2) fine mapping of breakpoints in genomic deletions/ duplications; and (3) genotyping.

1Mb array CGH

Primary genomic screening for CNVs was performed with a modified array CGH platform from the donated 1 Mb BAC/PAC clone set of Sanger Institute (Hinxton, UK). This homemade array CGH with an approximate 1Mb resolution was used for analysis of patients with complex phenotypes and an abnormal skeletal/limb development. The

Table 2. Primers for RT Q-PCR analysis of RNA

No	ID	Forward primer 5'→3'	Reverse primer 5'→3'
		Chromosome 2p12	
1	*LRRTM4*	CGCAGCTGAAAGGCATGA	AGCACCCGTGAGCATAACAAG
2		GGGATCAGAATGGTGTGAATGA	TGGTCTCTTGTGCGGAAACC
		Chromosome 2q32.2	
3	*DIRC1*	GCTTCTCATCACCAGAAGGAAAA	TGGGCCTCAGGCATTTGT
		ACATCACTGCCCACCACAGA	TTGCTGAGTGGTGGCAAGTAA
4	*GULP1*	TGTGTGCGGGAGGGAACT	CAAGTCCCATTCAACATCGTAAAC
		CTAAGTCGCCCTCCACTGACA	GGCATCGAAGACTGGTGTGA
5	*WDR75*	TTTCAGTGACAGGCACCAGTCT	TGCATCGCGCCACTCTAC
6		GGTGCTACTGAAAATGGCATTCT	TGCATTCCACTCCAATGCA
7		CATACCCGCAATTAGTGAGCTTCT	GAGCACAGGAAAGCAGCAGAT
8	*ORMDL1*	CCCACAGTGAAGTGAATCCAAA	CCAATGCATATGTCAGCCACAT
9		CAGGTCTGCAGTAAAAGATAACATTTTC	ACCCACGTATTCAAAGGCAAA
10	*PMS1*	CTTGCGGCTAGTGGATGGTAA	TTTCGCTTTTAACAGAGCAGCTT
11		AACAAGGCAGTTATTTGGCAGAA	GCAGTCCCCAGAACTGACATG
12		AAATGTCACTAAAAGATGGCAGAAAA	CTTGTGCTTCTGGGCCAAA
13	*GDF8*	AGTATGATGTCCAGAGGGATGACA	TTGTTTCCGTTGTAGCGTGATAA
14		CCCAGGACCAGGAGAAGATG	AAATCCCTTCTGGATCTTTTTGG
		Chromosome 13q14.2	
15	*LRCH*	AACCTGAGCGCCAGGAAAT	CCGTGTCCGAGAGGTCATG
16		CGACGACTGCAAAGTGTATGC	GGCACTGCTGCTGGCACTA
17	*Est D*	TCGGCCCGCTTCTTCTG	TTTTTGCTTGCACCAAATGG
18		AGCAATTGGACAGGAAAAGAATG	TGCAATCCCCCAAAGCA
19	*SUCLA2*	TGTGGAATCAGCAGCAGAAAA	TCTATCATGGTTGCATCGTATTTCA
		TGGACCCAGGAAGATGAAAGG	TTTCCATCGAGGCCAATGTAG
		TGCTGCTAAGGCAAATCTCAAC	GCCAAACCAGCACCATTTACTAG
20	*VDRIP (=MED4)*	GCTGCTGTCTGCGCTTGAG	TTCTTGAAATTGCCAGCATTTCT
21		GGGTTACTGGGTCAGATGAACAA	GCAAGTGCATCTCCTGGTAAATG
22		TCCACTGACCTGGGTTCCA	CCAGTAACCCACTTCTCATCTCTAAA
23	*ITM2B*	CCGCGCCATGGTGAA	TCGTCCTTCTTGGCCTCCTT
24		CAGAAACGTGAAGCCAGCAA	TTAAAGTTTCCACGGCAAATTTG
		Chromosome 18q23	
25	*HsG10342*	GGTGTGCGGGCTCATCTG	TCACGGGAAGCACGATCTACT
26		TCGCTGCTGCCAATGACA	CGATGGCCTCCATGATCCT
27		TGGGCGCCAGGACATCT	AGATGTGGGTCCTTCACAGAGAA
28		TGGGCGCCAGGACATC	ACATCATGACCACGAGCAGAT
29	*SALL3*	CCAAAGCACTCCTAGCCTGATC	TGACCGTTCACTTCCATTTTGA
30		GCAGCTCGGGCAATGAAC	TGGCGAGCCCGTTAGTGA
		Chromosome 15q13.3 (*GREM1-FMN1*) locus	
31	*GREM1ex2*	GCAAAACCCAGCCGCTTAA	GGTTGATGATGGTGCGACTGT

Table 2 continued

32	*FMN1ex15*	CAAAGTCTGGTGAGAAGGAGATCA	TTGTCTTGAAGTCACTGCAGAACTC
		reference genes	
33	*ACTB1*	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
34	*ACTB2*	CACCCTGAAGTACCCCATCG	TGCCAGATTTTCTCCATGTCG
35	*B2M*	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
36	*CLK2*	TCGTTAGCACCTTAGGAGAGG	TGATCTTCAGGGCAACTCGGG
37	*GAPDH*	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
38	*GUSB*	AGAGTGGTGCTGAGGATTGG	CCCTCATGCTCTAGCGTGTC
39	*UBC*	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
40	*YWHAZ*	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT

hybridizations and the calculations were done as previously described (Vermeesch *et al*., 2005; Menten *et al*., 2006). A loop-design was used for all experiments allowing dye-swap analysis replicates at substantially reduced costs per patient. Thus, three differentially labelled patient's samples were two-times hybridized in a way that any one was combined against the two others (Allemeersch *et al*., 2009).

Full tiling-path array CGH

Fine mapping of the breakpoints in patients with 2q31 deletions was done using chromosome 2 specific tiling path BAC array. The 32K BAC clone set was donation of BACPAC (BPRC) Resource Centre at Children's Hospital Oakland Research Institute (BPRC-CHORI, Oakland California, USA) (Krzywinski *et al*. 2004). All hybridizations with dye-swap replicates and the analyses were done as described (Van Buggenhout *et al*., 2005). The size of the detected aberrations is the maximum one defined by the first flanking BAC clones on the tiling-path array CGH platform for which a normal log2 ratio was found.

Affymetrix 250NspI array CGH

Fine mapping of the chromosome 15q13.3 CNV breakpoints was performed with the Affymetrix 250K NspI oligoarray platform (Affymetrix, Santa Clara, California, USA). Samples were processed in accordance to the manufacturer's instructions. In brief, 250ng of genomic DNA was digested with Nsp1 and after ligation to adaptors, amplified using generic primers recognizing the adaptor sequences. Subsequently 90µg of PCR product was fragmented and labelled with

biotin. All hybridizations were performed in an Affymetrix GeneChip Hybridization Oven and the arrays were washed and stained in the Affymetrix GeneChip Fluidics Station 450. Array scanning and image analysis were performed with Affymetrix GeneChip Scanner and Affymetrix GeneChip Operating Software v1.4 (GCOS), respectively. Genotype calls were obtained with Affymetrix Genotyping Analysis Software v4.1 (GTYPE) using the BRLMM algorithm. CNV analysis was performed by Affymetrix Chromosome Copy Number Analysis Tool v.4.0.1 (CNAT) using as control 41 female HapMap CEU individuals.

Agilent 244K array CGH

For fine mapping of the 10q24 duplication/ triplication breakpoints, the 244K oligoarray (Agilent, Santa Clara, California, USA) was used. All hybridizations and the analysis were performed according to the manufacturer's protocol with small modifications (Thienpont, 2009) using a circular binary segmentation (CBS) algorithm for aberration detection, and a smoothing window of 10 points (Venkatraman and Olshen, 2007). The arrays were scanned with Genepix Axon 4000B (Molecular Devices, Sunnyvale, California, USA). The feature extraction from the scanned slides was done with the Agilent Feature Extraction Software v.9.5.3.1. (Agilent, Santa Clara, California, USA). The extracted data were processed with CGH-v4_10_Apr08 or CGH-v4_95_Feb07 protocol using the provided slide and probe annotation files (Agilent, Santa Clara, California, USA). The CBS method was described by Venkatraman and Olshen (2007) and we used this hybrid method for p-value calculations of our data. It was implemented in R using the following settings: a=0.002, 5000 permutations. Splits were undone if they differed less than the 0.1 times the standard deviation (calculated after a 0.05 trim of the input data). Four abnormal consecutive clones were used to call a duplication, allowing 3.5% false negative results (Thienpont, 2009).

PCR amplification and direct sequencing

The coding part of the *GDF6* and *GREM1* exons, including the intron-exon boundaries, was PCR amplified from genomic DNA. In addition, to look for mono-allelic expression on cDNA level, two genes *Esterase D* and *WDR75* were sequenced for known coding SNPs (dbSNP). Gene specific primers were designed using RepeatMasker pre-analyzed sequences as an input for the Primer3 and/ or ExonPrimer software (Table 3). Subsequently, the amplicons and the

primer specificity were checked *in-silico* by BLAST, BLAT and UCSC *in-silico* PCR (Altschul *et al*., 1990; Kent, 2002; Karolchik *et al*., 2003; Johnson *et al*., 2008) against the NCBI Build 36.1 reference human genome sequence. For the amplification 100-150ng genomic DNA or cDNA were used with 0.2µl Taq polymerase (50U/µl, Roche Diagnostics GmbH, Penzberg, Germany), 5µl 10xPCR buffer (Roche Diagnostics GmbH, Penzberg, Germany), 5µl dNTPs (2µmol) and sterile water up to 50µl total reaction volume. The thermo-cycling conditions were adjusted according the primer specifications. After purification of the PCR product with ExoSAP IT (Roche), sequencing was performed with BigDye Terminator cycle sequencing kit on ABI 3100 Avant system (Applied Biosystems, Foster City, California, USA). The output sequence reads were analyzed with Seqman software (DNA star, Konstanz, Germany).

Table 3. Primers for PCR amplification and direct sequencing

No	ID	Forward primer 5'→3'	Reverse primer 5'→3'
	primers for direct sequencing of the coding part of *GREM1*		
1	*GREM1ex2_1*	TGCGTTTAAATGCTAGGTGC	GAAGCGGTTGATGATGGTG
2	*GREM1ex2_2*	CAAGAGGCCCTGCATGTG	TCTGGTTGTTTTAGGTCTGGG

Web-based electronic sources:

BLAST: http://blast.ncbi.nlm.nih.gov/

BLAT: http://genome.ucsc.edu/cgi-bin/hgBlat?command=start

BPRC-CHORI: http://bacpac.chori.org/home.htm

Ensembl Genome Browser: http://www.ensembl.org/index.html

ExonPrimer: http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html

GeneCards: http://www.genecards.org

GeneSeeker: http://www.cmbi.kun.nl/GeneSeeker

GEO: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geo

HomoloGene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=homologene

MGI: http://www.informatics.jax.org/

NCBI: http://www.ncbi.nlm.nih.gov

OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

Primer3: http://frodo.wi.mit.edu/primer3/

PubMed:	http://www.ncbi.nlm.nih.gov/pubmed/
RepeatMasker:	http://www.repeatmasker.org/
UCSC Genome Browser:	http://genome.ucsc.edu/
UniGene:	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene
UCSC:	http://genome.ucsc.edu/
VISTA:	http://genome.lbl.gov/vista/index.shtml

4. RESULTS

Chapter I. Positional cloning of candidate genes in apparently balanced chromosomal aberrations

Reciprocal translocations are rearrangements that result from a single break in each of the two participating chromosomes. All chromosomes have been reported to participate in reciprocal translocations. They occur in about 1 in 625 individuals in the population and most of them are considered "private" as they are only found in members of the same family (Gardner and Sutherland, 2004).

Inversions are intrachromosomal structural rearrangements. The most common are the simple (or single) inversions. If the inversion coexists with another rearrangement in the same chromosome it is a complex inversion. The simple inversions comprise a two-breakpoint event involving just one chromosome. If the inverted segment includes the centromere, the inversion is pericentric, if not it is paracentric.

Balanced chromosomal rearrangements may be either familial or *de novo*. Warburton (1991) estimated that 6.7% of the *de novo* reciprocal translocations and inversions are associated with phenotypic abnormalities. However, there could be an underestimation of the causal genotype/ phenotype relationship since there are no data about long term follow-up of "phenotypically normal" carriers for the presence of late onset diseases which remained undetected at the time of the cytogenetic finding (Bache *et al*., 2006).

A number of hypotheses have been postulated to explain the observed phenotypic abnormalities: (1) a direct disruption of gene(s) at the aberration breakpoint(s), with loss of function of the affected transcript(s)/ protein(s); (2) a position effect of the cytogenetic rearrangement upon the function of neighbouring gene(s); (3) the presence of cryptic (cis- or trans-), undetected cytogenetically deletion(s)/ duplication(s) leading to an abrogated function of gene(s); (4) unmasking of a recessive allele; and (5) uniparental disomy if the locus is known to be imprinted. Finally, there may be no causal relationship between the patient's phenotype and the balanced chromosome rearrangement (Astbury *et al*., 2004).

Over the years, mapping of translocation breakpoints in patients proved to be a straightforward method for the identification of new disease genes (Bugge *et al*., 2000). The number of published *de novo* and familial chromosomal rearrangements associated with phenotypic abnormalities is gradually increasing, thus providing more candidate loci for the corresponding phenotypes. Nowadays, the combined use of classical high-resolution G-banded karyotyping and new molecular

cytogenetic techniques resulted in faster investigation of these chromosomal aberrations at a relatively low cost. This is possible thanks to the availability of the reference Human Genome Sequence and the utilization of human genomic libraries of bacterial artificial chromosomes (BACs), fosmids and other vectors derived from the Human Genome Project (Krzywinski *et al*., 2004). These sources in conjunction with classical FISH analysis and new techniques such as array CGH (Backx *et al*., 2007; Gribble *et al*., 2007), chromatin conformation capture on chip (4C) (Simonis *et al*., 2009) and new generation sequencing (Chen *et al*., 2008) enable more in-depth characterization of a series of patients, providing potentially interesting phenotype/genotype correlations (Vandeweyer and Kooy, 2009). Unfortunately, time constraints, and more importantly the limit of available reliable methodologies for further evaluation of candidate genes, especially in cases where no directly affected transcript by the translocation(s) is present, still remain a restricting issue.

In summary, chromosomal aberrations are a major cause of congenital malformations in man. The study of these cytogenetic abnormalities may lead to the discovery of new disease gene(s) or to the identification of novel pathogenic mutations in already described gene(s).

Part 1. Mesomelic form of chondrodysplasia and congenital glaucoma associated with *de novo* translocation (13; 18)(q14; q23)

Adapted from Genetic Counseling 2004;15:191-197

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Introduction

The skeletal dysplasias are a large, heterogeneous group of genetic conditions characterized by abnormal development, growth and maintenance of the human skeleton. The overall incidence rate of congenital anomalies affecting the skeleton reaches almost 16 per 10000 births and congenital skeletal dysplasias represent less than one third of this group (Stoll *et al*., 1989; Rasmussen *et al*., 1996; Dimitrov *et al*., 2000).

There are at least 200 well-recognized forms of bone dysplasias and skeletal dysostoses, which can be differentiated by clinical, radiological and genetic features. Many result in a deformity of skeletal cartilage or bone, leading to common features such as short stature, limb malalignment, muscle hypotonia and joint contractures. Non-skeletal cartilage may also be involved, resulting in upper airway or respiratory disorders in infancy, hearing defects, dental and ocular pathology.

In the past, several well distinct mesomelic forms of disproportionate short stature have been described, e.g. Dyschondrosteosis (Leri-Weill), Langer type (homozygous dyschondrosteosis), Nievergelt type, Kozlowski-Reardon type, Reinhard-Pfeiffer type, Werner type, dominant and recessive Robinow type, Mesomelic dysplasia with synostoses, Mesomelic dysplasia Kantaputra type, Mesomelic dysplasia Verloes type, Mesomelic dysplasia Savarirayan type. In addition, a few less delineated sporadic patients and familial observations of mesomelic dysplasias were published (Ventruto *et al*., 1983; Fryns *et al*., 1988; Brodie *et al*., 1998; Kerner *et al*., 1998; Nishimura *et al*., 1998; Kozlowski and Masel, 1999; Kitoh and Lachman, 2001; Camera and Camera, 2003).

We report the clinical and cytogenetic data in a female patient presenting an unusual form of mesomelic skeletal dysplasia with other systemic anomalies and an associated *de novo* translocation (13;18)(q14;q23).

Clinical report

This Bulgarian girl, product of an uneventful second pregnancy to young unrelated and healthy parents, was referred to the Clinical Genetic Unit at the age of one month because of an atypical form of skeletal dysplasia. Birth weight, length and head circumference were 3000g (SDS= -0.73), 46cm (SDS= -2.16) and 36cm (SDS= 1.22), respectively. The family history was unremarkable except that the mother had had one miscarriage.

Clinical follow-up noted a disproportionate mesomelic short stature and developmental delay (Figure 1A-G). There was mild facial dysmorphism with a high forehead, fine and sparse hair, hypertelorism, greyish-blue sclerae, midface hypoplasia, with de-

Figure 1. A, B, C, D, E, F & G: The phenotype at different ages: at 1 month of age (A & B); at 1 year of age (C, D & E); at 2,5 years of age (F & G). Note the midfacial hypoplasia, high promonent forehead, fine and sparse hair, downslanting palpebral fissures, small mouth, mesomelic shortening of the upper limbs and ulnar deviation of the hands.

pressed nasal bridge, down-slanting palpebral fissures (also seen in the mother), short up-turned nose, and long philtrum. Short forearms, ulnar deviation of the hands, fifth finger clinodactyly, varus position of the feet, bilateral sandal-gap, persistent mild muscular hypotonia with an open mouth and a low, hoarse voice were also present. Ultrasound screening revealed patent ductus arteriosus and an ophthalmologic examination revealed bilateral glaucoma with opaque corneal stroma, and a shallow anterior chamber.

At the age of two years (Figure 1C-E) surgical correction of the heart defect was performed. A month later, two episodes of convulsions were observed. Brain CT scan did not detect any abnormalities but the seizures persist until the age of nine years.

Serial skeletal radiographs showed progressive shortening of the forearms with distally hypoplastic ulnae and curved, proximally subluxated radii (Figure 2A,B). Dysplastic and irregular metaphyses were noted. The ulnae were more severely affected and a delayed

Figure 2. Upper limb radiographs at 1 year of age (A- AP position): there was striking shortening of the forearm bones (radiograph of right upper limb- AP position). The ulna was shorter with distally hypoplastic cup-shaped metaphysis. There were also bowing of radius and ulnar deviation of the hand; B) At 2.6 years of age radiographs of the left upper limb (B- AP position) detected delayed bone age, clinodactyly, subluxated proximally radius and hypoplastic lateral part of the ulna as a result of metaphyseal dysplasia affecting preferably the distal part of the forearm bones.

bone age was present (Figure 2A). Vertebral abnormalities were not detected. No striking lower limb deformities were observed. However, mild shortening of the femurs, broad and short femoral necks and an irregular ossification of the distal tibia metaphyses were detected over the years of follow-up (Figure 3A,B). Serum alkaline phosphatase and electrolytes were repeatedly normal.

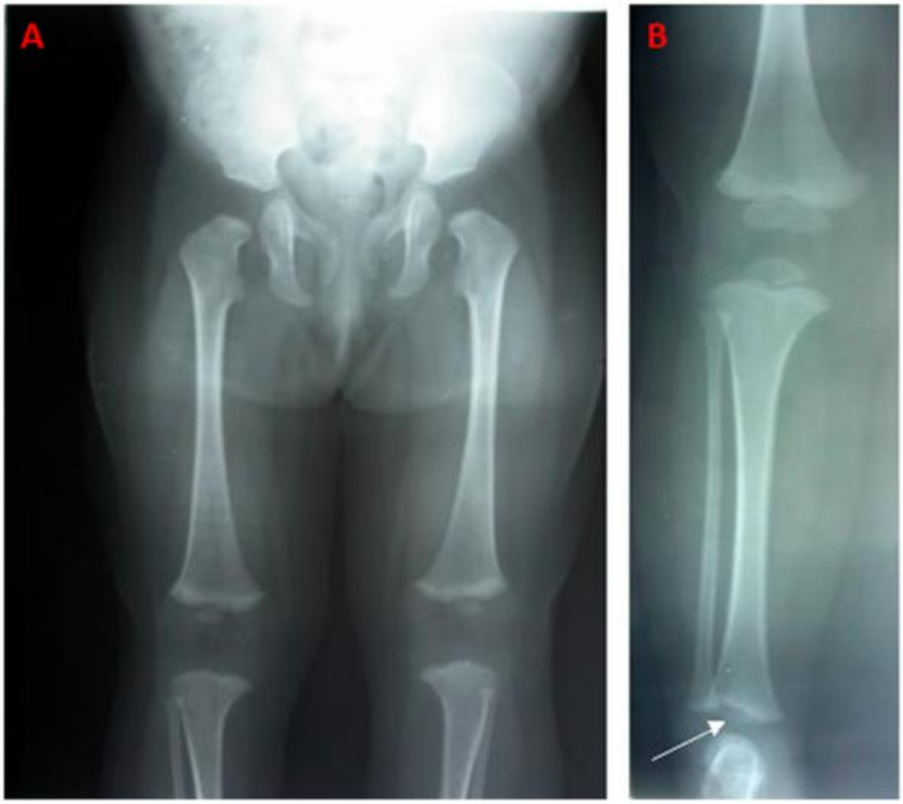


Figure 3. X-ray examinations of the lower limbs at 1 year (A- AP position) and 2.5 years of age (B- AP position) At 1 year of age (A) there was delayed ossification of the proximal femoral and tibial epiphyses. At 2.5 year of age (B) was detected irregular ossification of the distal metaphyses of the tibiae is noted (indicated with arrow).

High resolution G-banded karyotyping revealed a *de novo* reciprocal translocation (13;18)(q14;q23) (Figure 4).

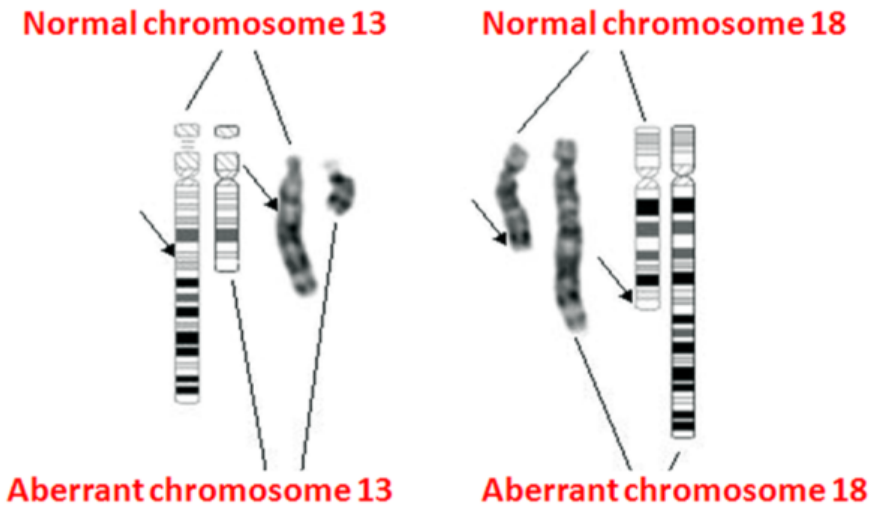


Figure 4. Partial karyotype and ideograms of chromosomes 13 and 18 showing a *de novo* translocation (13;18)(q14;q23). Arrows indicate the breakpoints.

Chromosomes 13q14 and 18q23 as candidate loci for an apparently new form of mesomelic chondrodysplasia

The present female patient has a mesomelic form of skeletal dysplasia, congenital glaucoma, cardiac defect and muscular hypotonia. In addition she carries a *de novo* reciprocal translocation (13;18)(q14;q23).

Based on the clinical and radiological findings, we could not classify the skeletal phenotype of this patient into one of the well-delineated syndromic or non-syndromic forms of mesomelic dysplasias (see also Table I).

There are a few other reports in the literature on not-well classifiable mesomelic shortening of the upper limbs. Fryns *et al.* (1989) published isolated mesomelic shortening of the forearms in father and daughter. More recently, Camera and Camera (2003) described another 5-year-old boy with mesomelic dysplasia affecting mainly the upper extremities. In both publications, the affected individuals had metaphyseal abnormalities similar to those observed in the present patient.

However, the present girl has additional ocular defects. Bilateral congenital glaucoma with shallow anterior chamber and opaque corneal stroma are seen in the Axenfeld-Rieger Malformation (ARM). The ARM syndrome is an autosomal dominant disorder characterized by abnormal development of the anterior segment of the eye and

subsequent glaucoma. There is genetic heterogeneity and at least 5 associated genomic loci exist. Most of the detected mutations affect PITX2 and FOXC1 genes (Lines *et al*., 2002). PAX6 mutations have been also reported (Rijse *et al*., 2001). Two additional loci are mapped at chromosome 13q14 (13) and 16q24 (Ferguson and Hicks, 1987; Werner *et al*., 1997). Interestingly, one of the two translocation breakpoints in our patient is 13q14. These data suggest that a gene(s) disrupted by the detected *de novo* translocation may explain her skeletal and/ or ocular abnormalities.

Table I. Differential diagnosis of the reported patient and some forms of mesomelic skeletal dysplasias

MSD / phenotype	Leri-Weill Dyschon-drosteosis	Langer type	Nievergelt type	Robinow type	Fryns 1983	Camera 2003	Present case
Age at onset	Late child	Birth	Birth	Birth	Birth	2 years	Birth
Short stature	Mild	Short stature	Short stature	Mild shortening	Normal stature	Normal stature	Short stature
Ulnae	Madelung deformity	Distal hypoplasia	Rhomboid	Distal hypoplasia	Short and thick	Short with distal metaphyseal irregularities	Short with distal metaphyseal hypoplasia
Radii	Short and curved	Short and deformed, proximal hypoplasia	Rhomboid, proximal dislocation	Short, proximal dislocation	Short, dysplastic and curved	Short with distal metaphyseal changes and proximal dislocation	Short with distal metaphyseal changes and proximal dislocation
Tibiae	Short	Short, proximal hypoplasia	Rhomboid	-	-	Distal metaphyseal ossification irregularities	Distal irregular metaphyseal ossification
Fibulas	Short	Proximal hypoplasia	Rhomboid	-	-		
Metacarpals/ tarsals	Short	-	Tarsal synostosis	-	-	-	-
Phalanges	Short	-	-	Clinodactyly	-	-	Clinodactyly
Other radiologic/ clinical changes	Cubitus valgus, coxa valga, elbow contractures	Mandibular hypoplasia, ulnar deviation of hands	Radioulnar synostosis, elbow contractures, clubfoot	Craniofacial dysmorphism, hypoplastic genitalia, vertebral and rib abnormalities	-	Prominent forehead, maxillary hypoplasia, elbow contractures	Prominent forehead, midface hypoplasia, patent ductus arteriosus, glaucoma
Gene	*SHOX*	*SHOX*	Not known	*ROR2* and *WNT5a**	Not known	Not known	t(13;18)(q14;q23)

- normal or no data; * *ROR2* and *WNT5a* mutations are found in AR and AD types of Robinow syndrome, respectively

Part 2. Physical mapping of the breakpoints in two patients: (1) a girl with mesomelic chondrodysplasia associated with *de novo* translocation (13; 18)(q14; q23) (Dimitrov *et al*., 2004) **and (2) a female patient with a syndromic form of Klippel-Feil anomaly associated with *de novo* inversion (2)(p12q34)** (Papagrigirakis *et al*., 2003)

Introduction

Over the last years several approaches have been applied to identify genes involved in skeletal development and causing different bone dysplasias/ dysostoses. As was discussed above, one possibility is the study of *de novo* or familial chromosomal translocations associated with a distinct phenotype.

Two individuals with a specific phenotype associated with a *de novo* apparently balanced chromosomal aberration were identified. The first patient with mesomelic skeletal dysplasia (MSDP1), congenital glaucoma, congenital heart defect and a *de novo* translocation (13;18)(q14;q23) was described by Dimitrov *et al*. (2004). The second patient (KFP2) is carrier of a *de novo* inversion (2)(p12q34) associated with Klippel-Feil anomaly (fusion of 3-5th cervical vertebrae), developmental delay, short stature, facial asymmetry, hearing loss due to abnormal ossicles, hypodontia and hypothyroidism. She was the third child of healthy parents with a normal family history. At birth the mother and father were respectively 38 and 47 year old. Her clinical phenotype was published by Papagrigorakis *et al*. (2004) and provides a new candidate locus for the Klippel-Feil syndrome.

There was no known molecular defect for these two conditions. We hypothesized that a disrupted gene could explain the abnormal phenotypes. Positional candidate gene approach was used to identify these putative genes.

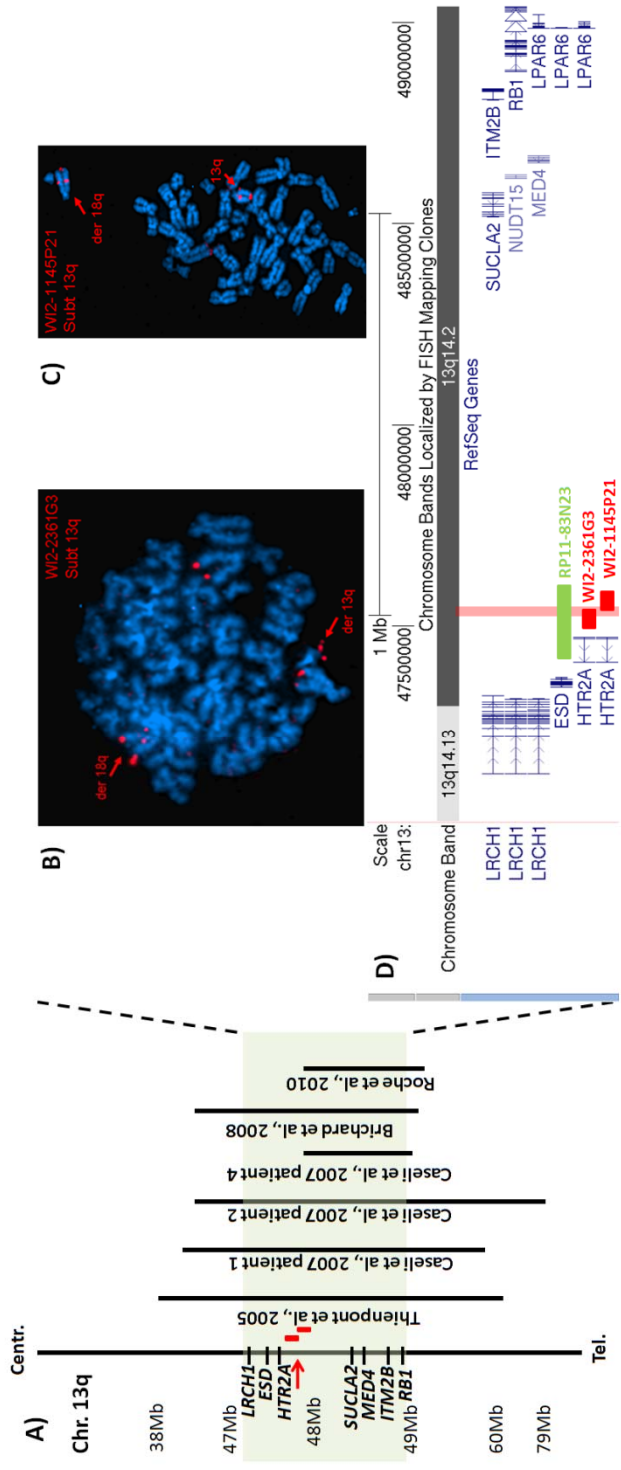
Results

Fine breakpoint mapping

Physical mapping of the four breakpoints to a resolution of 20-80Kb was carried out by "walking" FISH using commercially available BAC and fosmid clones as probes. In the first patient (MSDP1), two partially overlapping fosmids, WI2-2361G3 and WI2-1145P21, covered the breakpoint at chromosome 13q14.2 (Figure 1). For the second involved chromosome, there was a breakpoint spanning FISH probe WI2-414C22 at 18q23 (Figure 2). Similarly, in the second patient (KFP2) we were able to detect a split FISH signal with BAC

CTC-444N24 located at chromosome 2q32.2 (Figure 3) and the second inversion breakpoint at 2p12 locus was flanked by BAC RP11-9O10 and fosmid WI2-3162E4.
No genes were identified to be directly disrupted by the aberrations according the available data of the reference Human Genome Sequence (Ensembl, UCSC and NCBI) in these two patients. Several recent publications report on additional cis- and/ or trans-CNVs in carriers of apparently balanced chromosomal rearrangements. Therefore, a 1Mb array CGH analysis was performed but no additional loss or gain of genomic material was detected at this resolution.

Figure 1. A) Chromosome 13q (not drawn to scale) with genes located between 47-49Mb. The red arrow demonstrates the position of the breakpoint. Red bars represent the two fosmid clones that flank the breakpoint. Vertical black lines represent the size of 13q deletions in patients published by Thienpont et al. (2005), Caseli et al. (2007), Brichard et al. (2008) and Roche et al. (2010). The transparent green bar highlights the locus and genes analyzed for a possible position effect. B-C) FISH analysis results with the breakpoint-flanking fosmids WI2-2361G3 and WI2-1145P2 labelled in red are shown. The subtelomeric probe 13q (red signal) is used as control. D) Freeze of the UCSC genome browser (GRCh37/hg19) representing the highlighted area in part A. The transparent vertical red bar represents the position of the breakpoint. Horizontal red bars show the position of fosmids WI2-2361G3 and WI2-1145P2. There is no gene disrupted by the translocation.


A)
Chr. 13q
Centr.
Tel.
38Mb
47Mb
48Mb
49Mb
60Mb
79Mb
LRCH1
ESD
HTR2A
SUCLA2
MED4
ITM2B
RB1
Thienpont et al., 2005
Caseli et al., 2007 patient 1
Caseli et al., 2007 patient 2
Caseli et al., 2007 patient 4
Brichard et al., 2008
Roche et al., 2010
B)
WI2-2361G3
Subt 13q
der 18q
der 13q
C)
WI2-1145P21
Subt 13q
der 18q
13q
D)
Scale
chr13:
1 Mb
47500000
48000000
48500000
49000000
Chromosome Band
13q14.13
13q14.2
Chromosome Bands Localized by FISH Mapping Clones
RefSeq Genes
LRCH1
LRCH1
LRCH1
ESD
HTR2A
HTR2A
RP11-83N23
WI2-2361G3
WI2-1145P21
SUCLA2
NUDT15
MED4
ITM2B
RB1
LPAR6
LPAR6
LPAR6

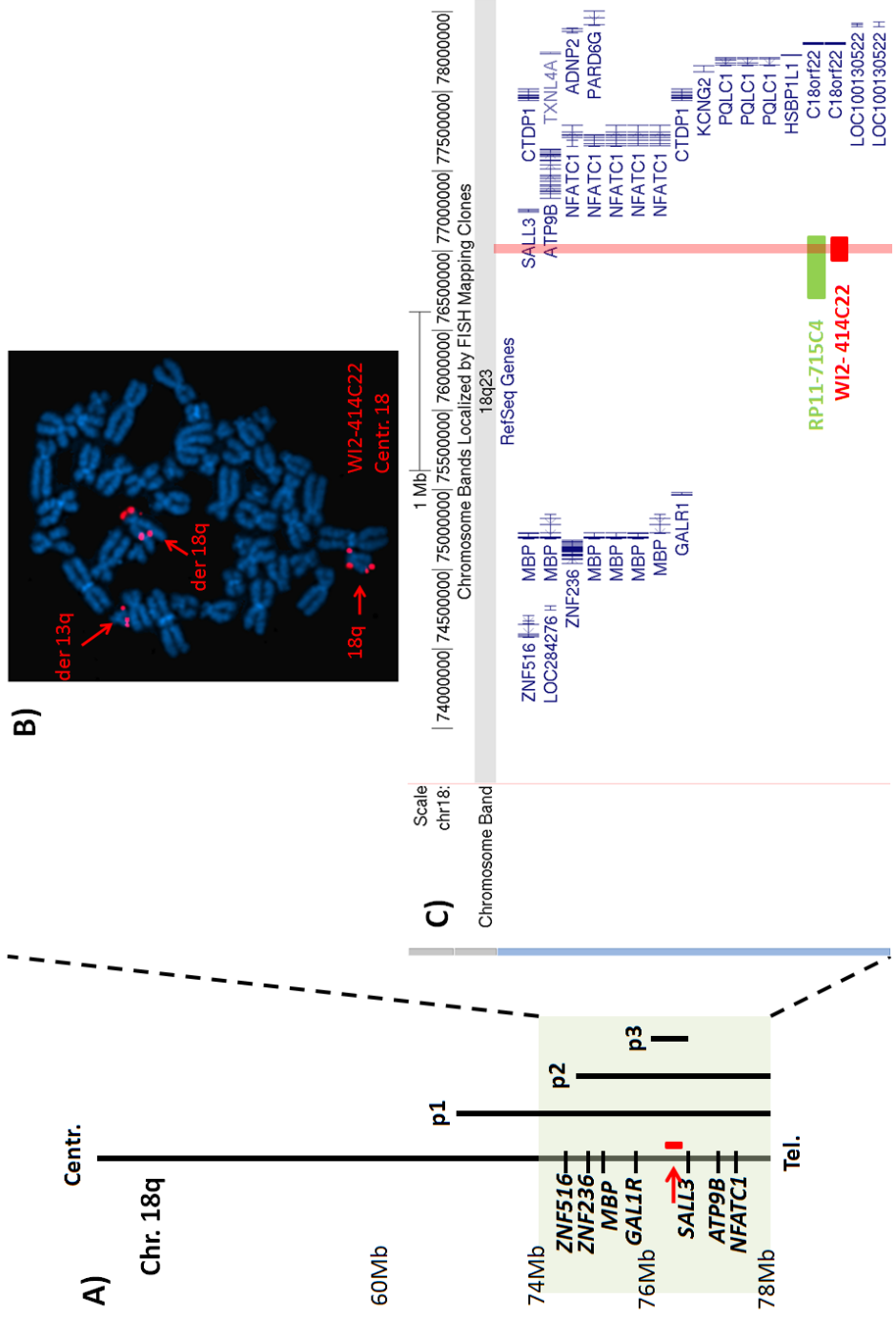

A)
Chr. 18q
Centr.
60Mb
p1
p2
p3
74Mb
76Mb
78Mb
Tel.
ZNF516
ZNF236
MBP
GAL1R
SALL3
ATP9B
NFATC1
B)
der 13q
der 18q
18q
WI2-414C22
Centr. 18
C)
Scale
chr18:
1 Mb
74000000 74500000 75000000 75500000 76000000 76500000 77000000 77500000 78000000
Chromosome Bands Localized by FISH Mapping Clones
18q23
Chromosome Band
RefSeq Genes
ZNF516
LOC284276
MBP
ZNF236
GALR1
SALL3
ATP9B
CTDP1
TXNL4A
NFATC1
ADNP2
PARD6G
KCNG2
PQLC1
HSBP1L1
C18orf22
LOC100130522
RP11-715C4
WI2-414C22

Figure 2. Chromosome 18q (not drawn to scale) with some of the genes located between 74-78Mb. The red arrow marks the position of the breakpoint. The red bar represents the breakpoint-spanning fosmid WI2-414C22. Vertical black lines, named p1, p2 and p3, represent the size of the 18q deletions in patients from our Centre. None of these patients have skeletal anomalies similar to MSDP1. The transparent green bar highlights the 18q23 locus. B) Fosmid WI2-414C22 (red signal) spans the breakpoint. A centromeric probe 18 (red signal) is used as control. C) Freeze of the UCSC genome browser (GRCh37/hg19) showing band 18q23. The transparent vertical red bar represents the position of the breakpoint. The horizontal red bar shows the position of fosmid WI2-414C22. There is no gene disrupted by the translocation.

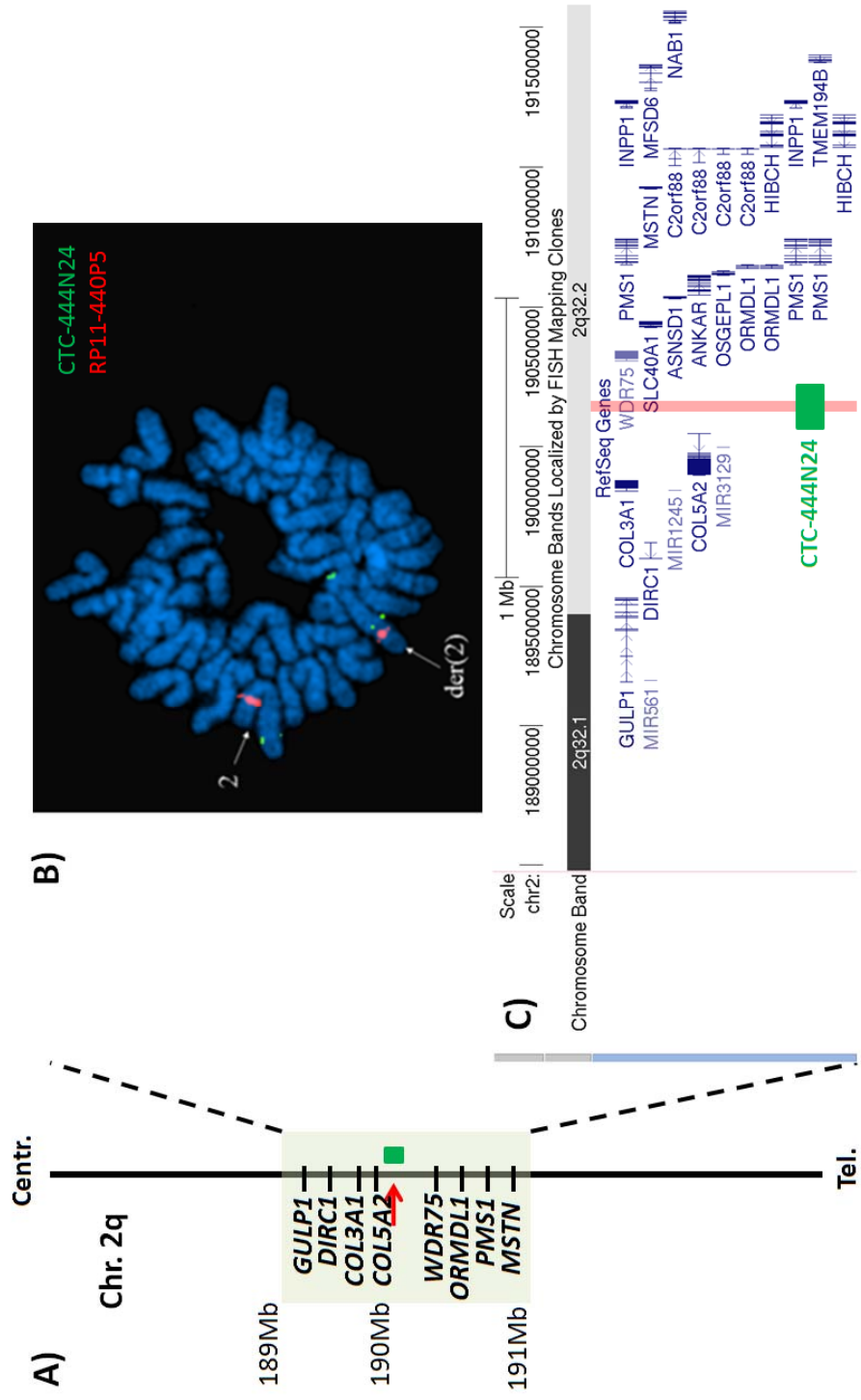

A)
Chr. 2q
Centr.
Tel.
189Mb
190Mb
191Mb
GULP1
DIRC1
COL3A1
COL5A2
WDR75
ORMDL1
PMS1
MSTN
B)
CTC-444N24
RP11-440P5
2
der(2)
C)
Scale
chr2:
1 Mb
189000000
189500000
190000000
190500000
191000000
191500000
Chromosome Band
2q32.1
2q32.2
Chromosome Bands Localized by FISH Mapping Clones
RefSeq Genes
GULP1
MIR561
COL3A1
DIRC1
MIR1245
COL5A2
WDR75
SLC40A1
ASNSD1
ANKAR
OSGEPL1
MIR3129
ORMDL1
ORMDL1
PMS1
PMS1
CTC-444N24
MSTN
C2orf88
C2orf88
C2orf88
HIBCH
HIBCH
INPP1
INPP1
MFSD6
NAB1
TMEM194B

Figure 3. A) Chromosome 2q (not drawn to scale) with some of the genes located between 189-191Mb. The red arrow marks the position of the breakpoint. The green bar represents the breakpoint-spanning BAC CTC-444N24. The transparent green bar highlights the 2q32.1q32.2 locus. B-C) A split signal of BAC CTC-444N24 (green signal) was detected. BAC RP11-440P5 (red signal) is used as control.C) Freeze of the UCSC genome browser (GRCh37/hg19) showing band 2q32.1q32.2. The transparent vertical red bar represents the position of the breakpoint. The horizontal green bar shows the position of BAC CTC-444N24. It lies almost 40Kbps downstream from *COL5A2* and does not cover any currently known genes.

Selection of "candidate gene(s)"

Computational analysis

To exclude/ confirm the possibility of a position effect, all genes located in a region of 1.5Mb up- and down-stream of the breakpoints were considered as "putative" candidate genes. Based on an evaluation of their potential to represent a "reliable candidate gene", fourteen transcripts in the vicinity of the translocation breakpoints were selected for further evaluation. Literature review (PubMed), expression profiles (GEO, UniGene), known or suspected biological functions (HomoloGene) and known associations with human or animal disorders (OMIM, OMIA, MGI) were the major criteria for selection. The restriction of our interest to transcripts with "proven" current status in the three major human genome browsers (Ensembl, UCSC and NCBI) was based on observations that the maximal distance of a position effect described thus far is approximately 1.3Mb (Velagaleti *et al*., 2005). In addition, terminal deletions of chromosome 18q are frequent and often include band 18q23. None of the currently known individuals with the 18q deletion syndrome feature skeletal abnormalities consistent with a diagnosis of mesomelic skeletal dysplasia. Thus, the involvement of a gene at chromosome 18q23 as a cause of the skeletal phenotype in the first patient (MSDP1) is unlikely. Therefore, the investigations at this locus were restricted to two transcripts, *HsG10342* and *SALL3*.

Finally, the following fourteen candidate genes were selected: (1) for chromosome 2p12 - *LRRTM4*; (2) for chromosome 2q34 – *DIRC1*, *GULP1*, *WDR75*, *ORMDL1*, *PMS1*, *GDF8*; (3) for chromosome 13q14 – *LRCH*, *Esterase D*, *SUCLA2*, *VDRIP* (=*MED4*), *ITM2B*; and (4) for chromosome 18q23 – *HsG10342* and *SALL3*.

Expression analysis of the "candidate genes"

The expression levels of the selected "candidate genes" were analyzed in EBV-cell-line derived cDNA (Table 1). Normal expression patterns were observed for *ORMDL1*, *GDF8, LRCH*, *SUCLA2*, *VDRIP, DIRC1*, *GULP, PMS1* and *ITM2B*. No expression was detected for *LRRTM4*, *HsG10342* and *SALL3*. For two genes, *Esterase D* and *WDR75,* there was a wide variability of the expression profile within the control samples. Therefore, it was impossible to conclude whether the expression was significantly disturbed in the patients. For these two genes, the mono-allelic expression of currently known coding SNPs was alternatively analyzed at cDNA level and no heterozygosity was detected (Table 1).

Table 1. Expression patterns of the selected for analysis genes.

gene	chromosome	distance to the breakpoint*	expression	
			RT-Q-PCR	SNPs
LRRTM4	**2p12**	**350 kbps (5')**	**no expression in EBV cell line**	
DIRC1	**2q32.2**	**500kbps (5')**	**no significant abnormalities in EBV cell line**	
GULP1	**2q32.2**	**700kbps (3')**	**no significant abnormalities in EBV cell line**	
WDR75	**2q32.2**	**160kbps (5')**	**high variability among the controls in EBV cell line**	**not detected**
ORMDL1	**2q32.2**	**490kbps (3')**	**no significant abnormalities in EBV cell line**	
PMS1	**2q32.2**	**500kbps (5')**	**no significant abnormalities in EBV cell line**	
GDF8	**2q32.2**	**780kbps (3')**	**no significant abnormalities in EBV cell line**	
LRCH	**13q14.2**	**200kbps (3')**	**no significant abnormalities in EBV cell line**	
Est D	**13q14.2**	**160kbps (5')**	**high variability among the controls in EBV cell line**	**not detected**
SUCLA	**13q14.2**	**1mbps (3')**	**no significant abnormalities in EBV cell line**	
***VDRIP* (=*MED4*)**	**13q14.2**	**1.1mbps (3')**	**no significant abnormalities in EBV cell line**	
ITM2B	**13q14.2**	**1.2mbps (5')**	**no significant abnormalities in EBV cell line**	
HsG10342	**18q23**	**directly disrupted**	**no expression in EBV cell line**	
SALL3	**18q23**	**150kbps (5')**	**no expression in EBV cell line**	

*- according the May 2004 Human reference sequence, NCBI build 35 (NCBI35/hg17)

Discussion

In both patients, the chromosomal aberrations did not disrupt any known genes. Moreover, we did not find any gross alterations in the expression pattern of all selected "candidate genes" in EBV-lymphoblastoid cell lines. The implementation of array CGH analysis in the clinical practice allowed the detection of an enormous number of genomic CNVs scattered throughout the entire human genome. Based on searches in databases with information reporting human

CNVs (Cartagenia, DECIPHER, ECARUCA) (Feenstra *et al*., 2006; Van Vooren *et al*., 2007; Firth *et al*., 2009) and review of the current literature we found a few individuals carrying deletions encompassing all four breakpoints that we mapped (Barber *et al*., 2005; Thienpont *et al*., 2005; Caselli *et al*., 2007; Brichard *et al*., 2008; Roche *et al*., 2010). None of these published patients had any skeletal defects. This excludes the possibility that a position effect and subsequent simple haploinsufficiency of genes in close proximity to the delineated breakpoints could explain the skeletal phenotypes in Patient 1(MSDP1) and Patient 2 (KFAP2). After these negative findings further analyses were not performed. However, we cannot firmly exclude or confirm the genotype/ phenotype relationship in the studied individuals. Of interest, *RB1* gene is located on chromosome 13q14.2. Individuals with disturbed *RB1* function as a result of gene mutations or genomic deletions have retinoblastoma (Thienpont *et al*., 2005; Caselli *et al*., 2007; Brichard *et al*., 2008). Recently it was demonstrated that Axenfeld-Rieger anomaly can also be observed in the 13q14.2 deletion/ retinoblastoma syndrome (Roche *et al*., 2010). The physical distance between the *RB1* gene and the 13q14 breakpoint in our patient with MSD and Axenfeld-Rieger-like ocular defects is approximately 1.5Mb. Therefore a hypothetical position effect upon the *RB1* function could explain at least partially the eye defect in this individual. No retinoblastoma has been detected in this Bulgarian girl.

The lack of explanation for the phenotypes in patients with balanced chromosomal aberrations and no directly disrupted gene is a well-known phenomenon (Kleinjan and Lettice, 2008; Spitz and Duboule, 2008; Vandeweyer and Kooy, 2009).

At present, only the abnormal patient's phenotype still suggests a suspected position effect. However, the full normal and abnormal phenotypic effect of the majority of the human genes remains unknown. Several recent publications demonstrate that genomic rearrangements affecting the regulatory landscapes of developmental master genes (e.g. *SOX9*) cause a spectrum of different developmental anomalies (Benko *et al*., 2009; Gordon *et al*., 2009; Kurth *et al*., 2009).

In addition, restricting the research interest of a possible position effect to the limit of its currently known maximum distance (e.g. 1.3Kb in *SOX9* gene), is imprecise and biased.

A general rule of thumb is that a position effect is due to a loss-of-gene-function. However, recent studies demonstrate more complex

interactions affecting the function of several contiguous genes in vicinity in the genome. Thus, the *ulnaless* (*ul*) strain if mice carry an inversion which disrupts the GCR-*Lnp-Evx2-Hoxd* regulatory landscape. This leads to both loss-of-function and gain-of-function tissue specific effects of different *Hoxd* genes in the developing limbs (Spitz *et al*., 2003).

Finally, currently there are technical limitations to the available approaches for prediction and analysis of regulatory sequences. This field is still under development (Poulin *et al*., 2005; Pennacchio *et al*., 2006; Prabhakar *et al*., 2006; Kleinjan and Lettice, 2008; Loots, 2008). Thus, it could be a daunting task to prove a position effect in early developmental genes due to: (1) limitation of patient samples, (2) dissimilarities with an inappropriately chosen animal model and (3) difficulties in the precise gene expression quantification during the development (Kleinjan and Lettice, 2008; Spitz and Duboule, 2008; Zeller *et al*., 2009). Hence, there is an urgent need for the development and application of new systems biology approaches to the study of developmental disorders.

Chapter II. CNVs as a cause of congenital skeletal defects

Genomic Copy Number Variations (CNVs) were defined as DNA segments that are present at a variable copy number in comparison with a reference genome sequence, e.g. deletions (homozygous or heterozygous), duplications, triplications, amplifications (Feuk *et al*., 2006; Zhang *et al*., 2009). The availability of new techniques for genome analysis resulted in the establishment of the concept of genomic disorders and led to explosion of new knowledge regarding the importance of structural genomic variations in human evolution, health and disease (Lupski, 1998, 2009). Even more, CNVs have been found to be implicated in the aetiology of complex traits such as autism, schizophrenia, epilepsy, Parkinson disease, Alzheimer disease, and emphysema. It was also demonstrated that they can behave as susceptibility factors for common disorders such as HIV infection, Crohn disease, glomerulonephritis, psoriasis, systemic lupus erythematosus. Finally, by changing our biased way of singe-locus thinking and thus the algorithm for genetic testing of patients with congenital anomalies of unknown aetiology, the analysis for CNVs helped to unravel the genetic cause of several anomalies affecting the skeleton and the limbs (Hurles *et al*., 2008; Conrad *et al*., 2010; Fanciulli *et al*., 2010; Stankiewicz and Lupski, 2010). In this project two different methods for CNV detection were used: quantitative PCR (RT Q-PCR) and array CGH analysis.

Part 1. RT Q-PCR analysis for detection of intragenic micro-deletions/duplications in patients with Holt-Oram syndrome

Holt-Oram syndrome (HOS) is an autosomal dominant condition characterized by upper limb radial-ray anomalies associated with cardiac septation/conduction defects. The most common molecular defects are mutations (~25-50% of the analyzed individuals) or intragenic deletions (~2% of the analysed individuals) of the *TBX5* gene (Borozdin *et al*., 2006; Debeer *et al*., 2007).
We identified sixteen presumptive HOS patients negative for *TBX5* mutations (Debeer *et al*., 2007). Strict phenotypical selection criteria were applied (McDermott *et al*., 2005). These patients were screened for intragenic deletions/ duplications (see Materials and Methods Table 1).
In two individuals intragenic deletions disrupting the *TBX5* gene were detected. The first patient (Figure 1) had a deletion of exon 8 and the other patient carried a deletion of exons 8-9. Both detected aberrations were *de novo*. No other CNVs affecting *TBX5* were found in the remaining fourteen cases. The detected two deletions preferentially

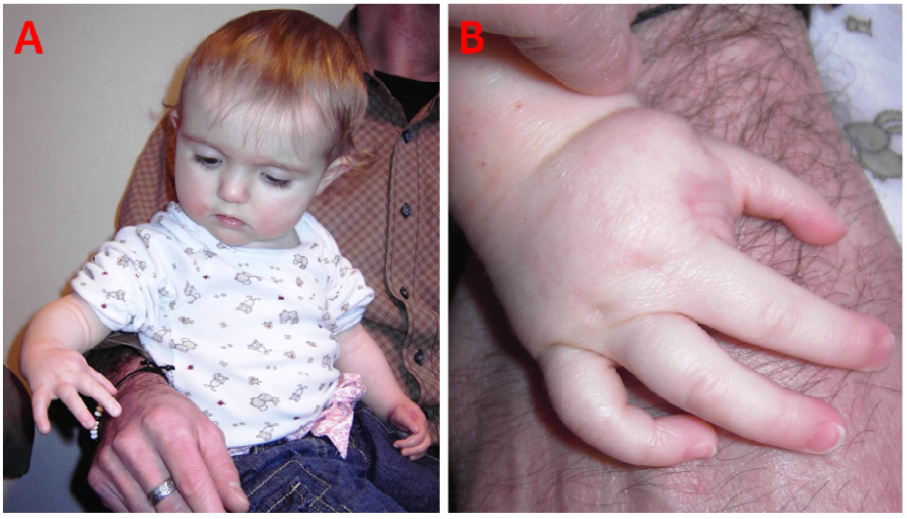


Figure 1. A-B) Patient 1 with a deletion of exon 8 of *TBX5*. Note the typical symmetric radial ray defects. In addition he has a congenital heart defect (VSD).

remove the last 3' *TBX5* exons similar to previous observations (Borozdin *et al*., 2006). There is no specific genotype/ phenotype correlation in comparison with patients with classic *TBX5* mutations (Debeer *et al*., 2007). Our data demonstrate a higher detection rate

(~12%) of intragenic deletions, confirming previous observations that strict clinical selection criteria improve the genetic testing yield (McDermott *et al*., 2005; Debeer *et al*., 2007). A substantial portion of patients with "classical" HOS or HOS-like phenotypes remain without known molecular defect, thus leaving room for new causal gene(s) to be discovered.

Part 2. Array CGH analysis of individuals with congenital skeletal anomalies

The CGH array is a relatively new, high throughput method for total genome screening in a single experiment. Given its enormous potential in identifying submicroscopic duplications/ deletions, it has already been implemented in the diagnostics of patients with developmental/ congenital abnormalities of unknown aetiology. This method has helped in identifying new genes causing inherited disorders (Vissers *et al*., 2004).

Forty-two patients with congenital skeletal anomalies as part of a syndrome, (e.g. Distal limb deficiency/micrognathia syndrome (OMIM 246560), syndromic forms of SHFM, polysyndactyly, multiple synostoses, Hajdu-Cheney syndrome, vertebral segmentation defects) have been selected for 1Mb array CGH analysis. Based on well-defined algorithm in this Centre for interpretation of CNVs (de Ravel, 2009; Thienpont, 2009), 10/42 (23%) causal deletions/ duplications were detected. The detected chromosomal aberrations and corresponding patients' phenotypes are summarized in Table 1. These results demonstrate the importance of array CHG in identification of the genetic aetiology of congenital skeletal defects. We were able (1) to extend the clinical spectrum of SHFM3, (2) to delineate two new genetic syndromes, and (3) to characterize further the phenotype of patients with 2q31 microdeletions and to define the critical loci for the observed limb/ skeletal defects and characteristic Facial Gestalt in the 2q31.1 microdeletion syndrome.

Table 1. Causal CNVs detected with array CGH at 1Mb resolution and associated clinical phenotypes

No	Patient ID	Phenotype	ISCN karyotype
1	C2755	DLDMS	46XY, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x4
2	C224256	DLDMS	46XX, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x3
3	C211000	DLDMS	46XY, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x3
4	C369373	iSHFM3	46XX, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x3
5	C369374	DLDMS	46XY, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x3
6	C365650	sSHFM3	46XY, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x3
7	C245063	iSHFM3	46XX, arr cgh 10q24.31q24.32(RP11-324L3→RP11-264H19)x3
8	C255625	ORUHRS	46XY, arr cgh 15q13.3(RP11-250G15)x0
9	C80286	CL-like OSD	46XY, arr cgh 15q13.3q14(RP11-250G15→RP11-3D4)x3
10	C267632	sS	46XY, arr cgh 1q24q(RP11-440G22→RP11-469A15)x1

DLDMS- Distal Limb Deficiency Micrognathia Syndrome; iSHFM3- isolated Split-Hand-Foot-Malformation 3; sSHFM3- syndromic Split-Hand-Foot-Malformation 3; ORUHRS- Oligodactyly, Radio-Ulnar Synostosis, Hearing loss, Renal defects syndrome; CL-like OSD- Cenani-Lenz-like oligosyndactyly; sS- syndromic form of symphalangism (extended phenotype and molecular data of this patient were published by Thienpont et al., 2009b)

Distal limb deficiencies, micrognathia syndrome (OMIM 246560) and syndromic forms of split hand foot malformation (SHFM) are caused by chromosome 10q genomic rearrangements

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Introduction

Split Hand Foot Malformation (SHFM) is a relatively rare anomaly characterized by central ray deficiencies and fusions of the remaining bones to variable degrees. Occasionally only remnants of the autopod are present (oligo/monodactylous ectrodactyly or peromelia). This condition represents 8-17% of all limb reduction defects and occurs in 1 in 20000 newborns (de Mollerat *et al*., 2003; Elliott *et al*., 2006). Non-syndromic and syndromic forms have been described and both can be familial or sporadic representing all possible types of inheritance. Reduced penetrance is often observed in affected families. Until now, five SHFM loci have been mapped in humans: SHFM1 at 7q21.2q22.1; SHFM2 at Xq26; SHFM3 at 10q24q25; SHFM4 at 3q27; and SHFM5 at 2q31. Only for one of them (SHFM4) the causal gene, *p63*, has been identified (Basel *et al*., 2006).

Using classical linkage analysis, the SHFM3 locus was originally mapped to 10q24, a region syntenic to mouse chromosome 19 (Ozen *et al*., 1999; Roscioli *et al*., 2004). The fact that the mouse *Dactylaplasia* phenotype, caused by insertional mutations of *Fbxw4*, resembles SHFM in man made the genes within the linkage region good candidates for the human phenotype (Sidow *et al*., 1999). No causal sequence alterations have been found, although two interesting genes (*FGF8* and *FBXW4*) reside within the critical locus. Intriguingly, a tandem duplication of 325-570Kb was detected in 17 familial and sporadic cases. All affected individuals had an isolated form of typical SHFM, except two. In one patient the phenotype was associated with medulloblastoma and another had a submucous cleft palate. Two genes, *BTRC* and *POLL*, were constantly included in the detected aberrations, whereas *FBXW4* was partially involved (de Mollerat *et al*., 2003; Basel *et al*., 2006; Kano *et al*., 2005; Everman *et al*., 2006; Lyle *et al*., 2006).

Selection criteria and patient's phenotypes

A group of individuals with a variety of syndromic forms of reduction limb defects were selected for genomic screening with a homemade

1Mb array CGH (Table 1). All patients were examined by experienced clinical geneticists and have normal high-resolution G-banded karyotypes. For two patients, C2755 and C224256, mutations in the coding part of *OSR1* and *OSR2* genes have been excluded by direct sequencing (Debeer *et al*., 2002a).

Family 1 (F1)

Case C2755, initially reported by Buttiens and Fryns (1987), is the second child of reportedly healthy unrelated parents. He has an affected younger sister (patient C224256). He presented with short stature, an asymmetric face, micrognathia, limited movements of the lower jaw, maxillary hypoplasia, high arched palate, severe myopia, high pitched voice, bilateral conductive hearing loss thought to be due to recurrent ear infections in childhood, oligomeganephronia leading to chronic renal insufficiency and severe, symmetric, terminal reduction limb defects affecting the hands and feet. He has mild to moderate mental retardation which is more pronounced than in his affected sister. X-ray examination revealed mid-facial hypoplasia and microretrognathia. There were severe truncation defects of the autopod, leading to a complete loss of both hands. Both radii were missing and the ulnae were hypoplastic and curved. The pre-axial part of the feet was more severely affected with many absent bones and synostoses of the remaining tarsal bones (Figure 1a-c) (Buttiens and Fryns, 1987).

Case C224256 is the younger sister of C2755. She presented with a similar, but milder facial and skeletal phenotype, and borderline to mild mental retardation. She had short stature, mild micrognathia and maxillary hypoplasia, high arched palate, and symmetric pre-axial reduction defects of the hands, affecting all four limbs. Both radii were mildly dysplastic and curved. The remaining skeletal structures were normal. Vision, hearing and kidney function were normal (Buttiens and Fryns, 1987).

Family 2 (F2)

Case C211000 is the second child of healthy, unrelated parents (C364835 and C364837). Clinical examination revealed short stature, micrognathia, mild maxillary hypoplasia, cleft palate, nystagmus and severe myopia, low set ears, preaxial reduction defects of all limbs with one remaining ulnar/fibular digit on both hands and feet, respectively. He had bilateral renal hypoplasia and progressive decreasing renal function leading to chronic renal failure necessitating

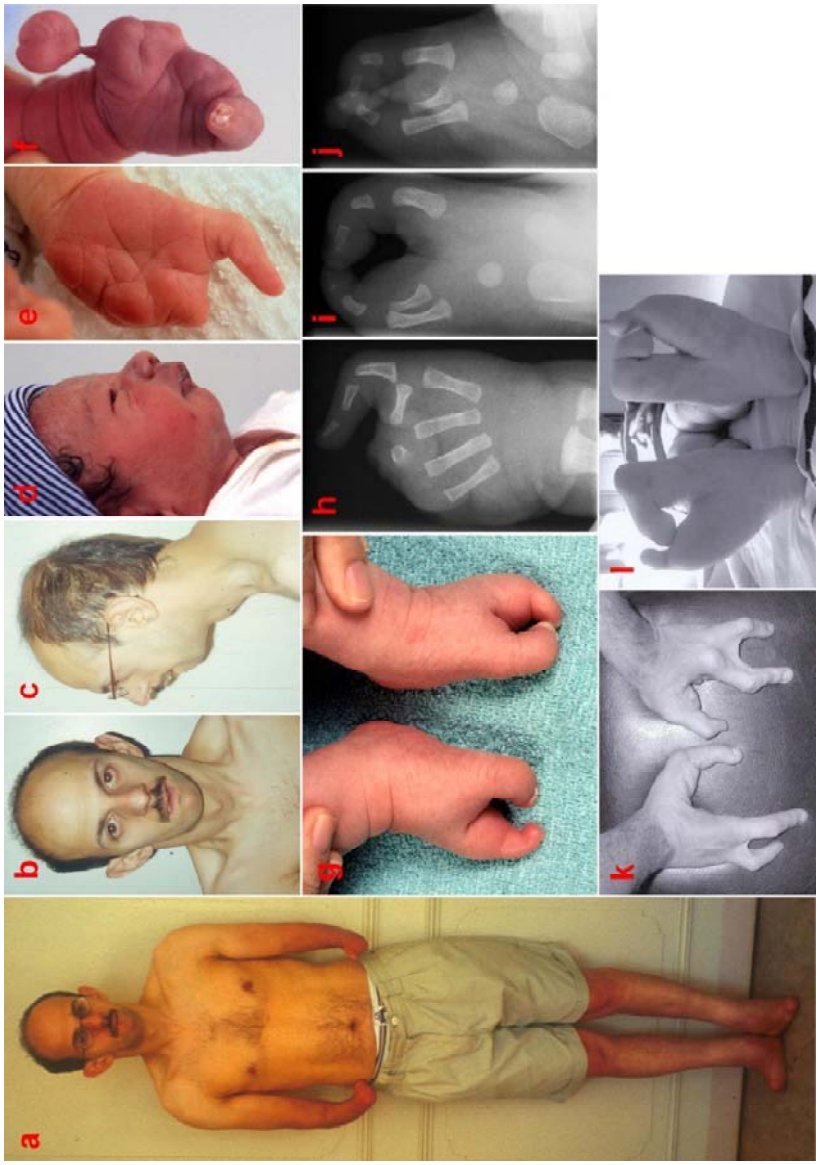


Figure 1. Clinical phenotypes of case C2755, C369374 and C365650. a-c) C2755 with LDMS originally published by Buttiens and Fryns (1987). All four limbs are symmetrically affected. There is marked facial asymmetry with pronounced micrognathia, maxillary hypoplasia and short stature; d-g) The facial gestalt of C369374 is similar to that of the affected siblings in Family 1- maxillary hypoplasia, micrognathia and low set ears. There are symmetrical terminal reduction defects of both hands with one ulnar finger remaining. An appendage like mass on the left hand is medially linked by a skin bridge to the palm. A large medial gap separates

the two digits of both feet; h-j) Hand and foot radiographs of C369374 demonstrate symmetrical terminal reductions of all four limbs. There are only four metacarpals and one ulnar (fifth) digit with three phalanges of both hands. In addition, an accessory short tubular bone pointing to the fifth metacarpo-phalangeal joint and one not very well defined ossification centre of bone remnant are present. Both feet have a classical SHFM phenotype with three metatarsal bones and two digits, each with phalanges. The last two metatarsals are fused at their distal ends and a big gap separates them from the first metatarsal. There is no ossification of carpal bones at that age and two tarsal bones are present; k and l) classic four limb SHFM phenotype of patient C365650.

a kidney transplantation. In addition, a bilateral 40dB conductive hearing loss due to recurrent ear infections was revealed. His phenotype is similar to those of the siblings in F1 (Keymolen *et al*., 2000).

Family 3 (F3)

C365650 is a patient with four limb ectrodactyly and renal hypoplasia (Figure 1k-l). His parents C381158 and C381159 are healthy and unrelated. He underwent a renal transplantation for chronic renal failure.

Family 4 (F4)

C369374 is the second child of healthy unrelated parents. Clinical examination revealed facial dysmorphism with remarkable micrognathia, limited movements of the lower jaw, midfacial hypoplasia and low set ears. He has severe preaxial reduction defects of both hands. On the right site there is one ulnar finger. On the left hand a medially placed rudimentary appendage that could be a remnant of a thumb, linked to the palm with a skin bridge and one wider ulnar finger with bifid nail are present. There is a classical split foot malformation bilaterally. X-ray examination of the right hand during his first year revealed four metacarpal bones, one ulnar digit with three phalanges, an additional phalanx, whose proximal end points toward the metacarpo-phalangeal joint of the single finger, and an ossification centre of a phalangeal remnant on the top of the second (from medial to lateral direction) metacarpal bone. There was no ossification of carpal bones. Both feet had two digits separated by a wide gap. The medial (first) ray had one metatarsal bone and two phalanges. The lateral ray had two metatarsal bones and one digit with three phalanges. Both talus and calcaneus and one additional tarsal ossification centre were also present. All long bones of the zeugopod were normal. Since the age of seven years on a progressive mixed

conductive-sensorineural hearing loss was detected (Figure 1d-j). There is history of recurrent ear infections. The skeletal defects, facial gestalt and detected hearing loss of this boy (C369374) resemble those of the patients with Distal limb deficiencies with micrognathia syndrome (DLDMS) (Buttiens and Fryns, 1987; Keymolen *et al*., 2000).
Patient C369373 is the younger sister of C369374. Classical SHFM of both hands and feet is present. She has no facial dysmorphism, particularly no micrognathia. Up to the age of six years no renal problems and no hearing loss were evident.
C369371 is the mother of C369373 and C369374, and is unaffected. X-ray examination of hands and feet did not reveal any skeletal abnormalities.
C369372 is the father of C369373 and C369374. He was born with oesophageal atresia. Clinical examination revealed micrognathia. No hand or foot malformations were noted clinically or radiographically.

Results

Detection of 10q24 CNVs

1Mb array CGH screening in a group of patients with unexplained syndromic forms of congenital skeletal anomalies revealed in two of them (C2755 and C224256) a gain of signal of three consecutive clones- RP11-324L3, RP11-529I10, RP11-264H19 (Supplemental Figure 1a,b). All three BACs were located on chromosome 10q24.31q24.32. The probands were originally published as having a new autosomal recessive syndrome (Buttiens and Fryns, 1987). Based on their phenotype- terminal limb reduction defects/SHFM in combination with facial dysmorphism and/or hearing problems, and/or renal insufficiency- three additional cases were selected for array CGH analysis. All patients carried an aberration of the same region.
In 4/5 analyzed individuals the Log2 ratio of the abnormal clones corresponded to a duplication. However, one patient (C2755), in contrast to his less severely affected sister, had a Log2 ratio consistent with a triplication (Supplemental Figure 1a).
Since apparently simple genomic rearrangements were often found to be more complex (Thienpont *et al*., 2008), the extent of the duplication and the presence of additional cis- and/or trans-copy number variations in this group of patients were further investigated. Therefore 10 individuals, including all affected members of the four families and the parents of one sporadic and one familial case, were analyzed with a 244K array CGH platform (Agilent).

Table 1. Patient's phenotypes.

Patients / Phenotype	C2755*	C224256*
Face	**maxillary hypoplasia, micrognathia, high arched palate**	**maxillary hypoplasia, micrognathia, high arched palate**
Eyes	**Severe myopia**	**normal**
Ears	**low set ears, recurrent otitis in childhood, bilateral conductive hearing loss**	**low set ears**
Upper limbs	**Absent radii, hypoplastic curved ulnae, rudimentary carpal bones, absence of other bones of the hands**	**deformation and bowing of distal radii, absent carpal bones, missing 1st right and 2nd right and 1st left metacarpals, one rudimentary dysmorphic bone between 3rd and 4th metacarpals, only one 5th finger with three phalanges**
Lower limbs	**normal long bones, bilateral synostosis of rudimentary calcaneus and talus, absent tarsal bones, one short and dysplastic tubular bone of both feet**	**normal long bones, talus, calcaneus, naviculare and cuboideum, rudimentary 1st metatarsal, missing 2-4 metatarsals, 5th metatarsal with large base and one two-phalangeal digit**
Internal organ abnormalities	**oligomega-nephronia**	**none**
MD	**moderate**	**Borderline**
OA	**undescended left testis**	**none**

MD- mental development; OA- other abnormalities; SHFM- Split Hand Foot Malformation; *- patients published by Buttiens and Fryns (1987); **- patient published by Kemolen *et al.* (2000).

C211000**	C369373	C369374	C365650
maxillary hypoplasia, micrognathia, cleft palate	normal	maxillary hypoplasia, micrognathia	normal
nystagmus, severe myopia	normal	normal	normal
low set ears, recurrent otitis, bilateral conductive hearing loss,	normal	low set ears, recurrent otitis, bilateral mixed conductive-sensorineural hearing loss	normal
normal long bones, two metacarpals, one postaxial (5^{th}) finger with two phalanges	classical SHFM	normal long bones, four metacarpals, one postaxial (5^{th}) finger with three phalanges, one phalanx crossing between 4^{th} and 5^{th} metacarpals and one rudimentary bone between 3^{rd} and 4^{th} metacarpals	classical SHFM
normal long bones, one metatarsal, one postaxial digit with one phalanx	classical SHFM	classical SHFM	classical SHFM
renal hypoplasia	none	none	renal hypoplasia
Normal	normal	normal	normal
None	none	none	none

No other common cis- or trans-copy number variations that can explain the more extended clinical features of our patients, have been detected. All affected individuals, except case C365650, carried a duplication of at least 500Kb, including five genes- *LBX1*, *BTRC*, *POLL*, *RP11-529I10.4* (*DPCD*) and *FBXW4*. Only C365650 had a larger duplication of 630Kb encompassing all genes from *TLX1* to *FGF8* (Figures 2 and 3).

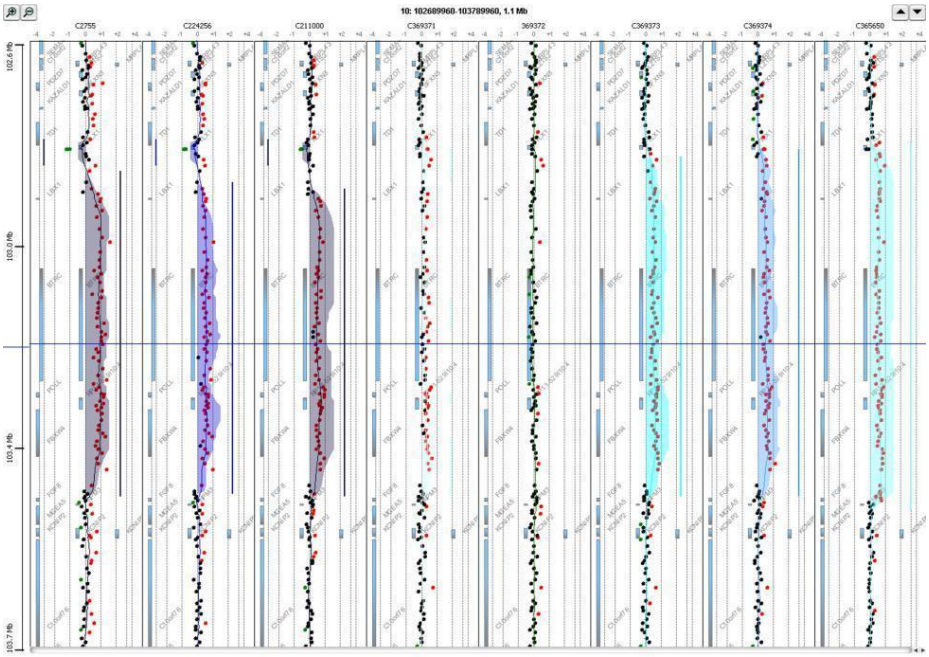


Figure 2. Agilent 244K array result of all six patients carrying a 10q duplication, C369371(the suspected of somatic mosaicism mother of C369373 and C369374) and the phenotypicaly normal father C369372 of the same siblings. For the exact position of the breakpoints and the size of the aberration in all four families see the text and Table 2. The severity of the phenotype corresponds to the colour marking the aberration- from greyish-blue as the most severe to light-blue as the less severe observed phenotype.

The proximal breakpoints were spread over an area of more than 100Kb, at least from probe A_16_P39153053 (102876069-102876128bp) to probe A_14_P139489 (102977418-102977470bp). In contrast, for five patients the telomeric ends of the duplication were clustered between probes A_16_P02330019 (103468797-103468856bp) and A_16_P02330056 (103498069- 103498128bp),

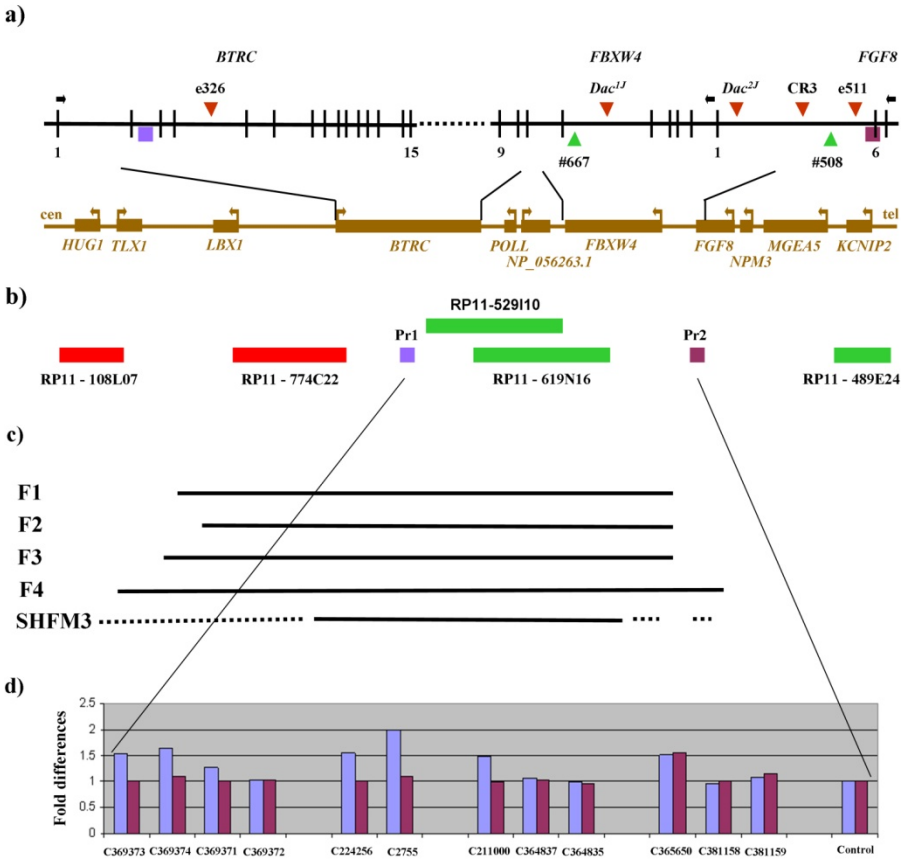


Figure 3. Organization of 10q24 duplication locus (not to scale). a) The brown colour line represents the genomic organization of the 10q24 locus duplicated in nonsyndromic and syndromic SHFM patients. Filled brown bars correspond to the genes and arrows indicate the 5' end of each of them. The black line above highlights some parts of the aberration- *BTRC*, *FBXW4* and the last two exons of *FGF8,* as well as the area between the last two genes. Vertical lines demarcate the exons of each pointed gene. The mutations in Dactylaplasia mice *Dac*^{1J} and *Dac*^{2J} are indicated with red triangles above the line (Kano *et al*., 2007). Blue and purple squares highlight the positions of Pr1 and Pr2, also shown in (b). Red triangles above and green triangles below the line demonstrate the position of regulatory sequences in mice and zebrafish, proven to drive the expression of reporter genes within the AER and in developing limbs (for CR3 see Beermann *et al*. (2006); for #667 and #508 see Kikuta *et al*. (2007); and for e326 and e511 see VISTA Enhancer Browser - http://enhancer.lbl.gov/frnt_page.shtml and Visel *et al*. (2007)); b) Red, green, blue and purple bars and squares present the position of BACs and primer pairs Pr1, and Pr2 used for FISH, and Q-PCR. They are aligned to the map shown in (a) but for their exact position see the text and Ensembl 52, Human Reference Sequence 2005, NCBI Build 36 - http://dec2008.archive.ensembl.org/ Homo_sapiens/Info/Index; c) Black lines F1, F2, F3 and F4 show the family specific

size of the 10q24 duplication with respect to the gene positions represented with brown bars in (a). For exact breakpoint positions see the text and Table 2. The last line named SHFM3 summarizes the duplication sizes of previously published cases. The dotted part of the line represents the different breakpoint positions as described (de Mollerat *et al*., 2003; Kano *et al*., 2005; Lyle *et al*., 2006); d) Q-PCR with primer pair Pr1 confirmed the presence of one additional copy in all affected individuals, except one. Only C2755 had fold difference of 2 using the same primers. This corresponds to the presence of a triplication in this individual. In addition, the healthy mother C369371 of C369373 and C369374 had fold difference of 1.25, which in addition to the FISH and array CGH data confirms that she is a carrier of somatic mosaicism. Q-PCR using Pr2 primer pair demonstrated a presence of one additional copy only in patient C365650.

except for C365650. In this case the distal breakpoint was flanked by A_14_P123126 (103524624-103524683bp) and A_16_P02330105 (103528589-103528648bp), thus suggesting that at least the last three exons of *FGF8* were also included in the duplication. All affected individuals from a same family shared the same breakpoints (Table 2). These data demonstrate that the observed rearrangement was similar in size to those previously detected in non-syndromic SHFM3 patients but extended more distally in the *FGF8* direction.

To confirm the array CGH results, FISH analysis with probes within and flanking the detected aberration was performed. Bacterial artificial chromosomes (BACs) were labelled with red and green dyes, respectively.

Three different combinations of labelled probes were hybridized on slides with spreads from cultured peripheral blood lymphocytes of C2755 and C224256 (Figure 4a-e). In concordance with previous data (de Mollerat *et al*., 2003), hybridization with combination of probes within the duplication (BACs RP11-744C22 in red and RP11-619N16 in green) on interphase nuclei from patient C224256 revealed presence of three red and three green signals. Four of the signals, two red and two green respectively, were grouped together. Their linear order red-green-red-green suggested a tandem duplication (Figure 4b). However, in the more severely affected brother C2755, FISH with the same probes detected four signals for each BAC, as was expected from the array CGH and Q-PCR results. Six signals, three red and three green, were always clustered but it was not possible to evaluate their relative position (in tandem or inverted orientation) (Figure 4c).

One explanation of the different phenotype in the syndromic SHFM patients could be the more complex character of the observed aberration, representing an insertion instead of simple tandem duplication. Thus the inserted fragment could disrupt a gene(s) in

Table 2. Results of genetic studies

Families	Individuals	Relatives	Phenotype	1Mb array	244K array (genomic position in bp)*	Q-PCR gCNs**	Reference
F1							
	C2755	brother of C224256	DLDMS	10q24 triplication	Chr10q 532.77Kb triplication (102965299-103498069bp)	4	Buttiens and Fryns, 1987
	C224256	sister of C2755	DLDMS	10q24 duplication	Chr10q 532.77Kb duplication (102965299-103498069bp)	3	Buttiens and Fryns, 1987
F2							
	C211000	two healthy siblings	DLDMS	10q24 duplication	Chr10q 528.72Kb duplication (102969344-103498069bp)	3	Keymolen *et al.*, 2000
	C364835	mother of C211000	healthy	na	normal	2	This study
	C364837	father of C211000	healthy	na	normal	2	This study
F3							
	C369371	mother of C369373 and C369374	healthy	na	Chr10q 597.29Kb duplication (102900770--103498069bp)	2.5***	This study
	C369372	father of C369373 and C369374	healthy****	na	normal	2	This study
	C369373	sister of C369374	SHFM	na	Chr10q 597.29Kb duplication (102900770-103498069bp)	3	This study
	C369374	brother of C369373	DLDMS	10q24 duplication	Chr10q 597.29Kb duplication (102900770-103498069bp)	3	This study
F4							
	C365650	none	sSHFM	10q24 duplication	Chr10q 658.43Kb duplication (102870153-103528589bp)	3	This study
	C381159	mother of C365650	healthy	na	na	2	This study
	C381158	father of C365650	healthy	na	na	2	This study

Legend to Table 2. SHFM- Split Hand Foot Malformation; sSHFM- syndromic SHFM; DLDMS- Distal limb deficiencies with micrtognathia syndrome; na- not analyzed; *- max size of the aberration according UCSC genome browser, Human Reference Sequence March 2006, NCBI Build 36.1.; **- present genomic copy numbers (gCNs) after RT Q-PCR analysis with Pr1; ***- carrier of somatic mosaicism; ****- surgically corrected congenital oesophageal atresia, micrognathia

vicinity thus contributing to the additional phenotypic features.
To further shed light on the genomic architecture, FISH experiments combining two additional BACs (RP11-108L07 labelled in red and RP11-489E24 in green) flanking the aberration were performed (Figure 4a). Depending on the combination, a string of two/three red or green signals followed by one red or green signal from the probe outside the rearrangement were always present (Figure 4d,e). Thus, the presence of an insertion was excluded.

Detection of somatic mosaicism for the 10q24 duplication in a healthy mother

Two of the cases were familial, each with two affected sibs and healthy parents, the other two were sporadic. Only in one familial case the parents were available for analysis. To further investigate the suspected somatic mosaicism of the phenotypicaly normal mother (C369371) of C369373 and C369374, a direct FISH analysis with two additional probe combinations was done. Each BAC combination, (1) RP11-774C22 labelled in red and PR11-489E24 labelled in green; and (2) RP11-108L07 labelled in red and RP11-529I10 labelled in green, detected a presence of 30% mosaicism in non-cultured peripheral white blood cells (Figure 4f,g).
Q-PCR assays were additionally carried out to confirm the array CGH result. Two primer pairs, one within BAC RP11-529I10 (Pr1) and another spanning the exon-intron boundary of exon 6 of *FGF8* gene (Pr2) were used (see Material and Methods Table 1). Twelve individuals were included in the analysis. Unfortunately the parents of the original probands (C2755 and C224256) were not available for further study.
In all affected individuals, except one, the Pr1 amplicon showed a 1.5 fold difference, corresponding to the presence of three copies in the analyzed genome. Only for C2755 a fold difference of 2 was detected suggesting the presence of a triplication, which is in correlation with the array CGH and FISH result. The unaffected mother (C369371)

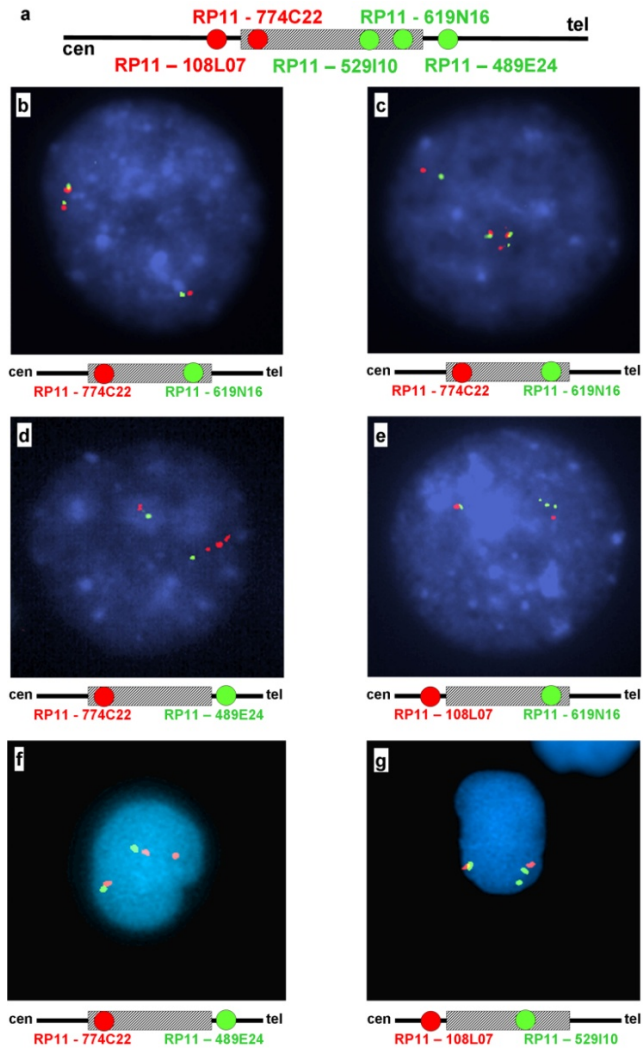


Figure 4. a) Five BACs, three within and two flanking the duplication, were labelled in red and green as shown. The dashed bar represents the aberration and the coloured circles the corresponding FISH probes. Below each individual experiment in (b), (c), (d), (e), (f) and (g) a similar schematic graph shows the position and colour of the used FISH probes; b) Interphase FISH analysis with probes RP11-774C22 (red) and RP11-619N16 (green), both within the 10q24 duplication, revealed the presence of a tandem duplication in C224256; c) The same combination of BACs demonstrated the presence of four hybridization signals for both probes in lymphocyte nuclei of C2755, her more severely affected brother. Three red and three green signals were always brought together, suggesting that they share one allele; d) Interphase FISH with combination of BAC RP11-489E24 (lying distally to the aberration) and BAC RP11-774C22 (within the rearrangement) showed the presence of four red signals and two green signal in C2755. A string of three red and one green and separately paired red and green signals were present in each screened nucleus; e) In the same patient (C2755), a similar result was obtained using a combination of BAC RP11-108L07 (proximal to the duplication/triplication) and BAC RP11-619N16 lying within the aberration; f-g) Direct FISH with two different probe combinations, (1) RP11-774C22 labelled in red and PR11-489E24 labelled in green, and (2) RP11-108L07 labelled in red and RP11-529I10 labelled in green, demonstrates the presence of a cell line with 10q24q25 duplication in individual C369371 (the normal cell population is not shown).

of C369373 and C369374 consistently showed a 1.24 fold difference. This could be explained if she is a somatic mosaicism carrier, this again in agreement with the 244K Agilent array CGH and FISH results (Figure 3).
All tested individuals except one, had one copy of the *FGF8* gene (Pr2). Only C365650 was a carrier of a duplication of at least the last sixth exon of the gene (Figure 3).

Discussion

Syndromic forms of SHFM are caused by a 10q24 duplication

In this study 10q24 duplications were detected in six patients with Distal limb deficiencies, micrognathia syndrome (DLDMS) or syndromic forms of SHFM. All cases had duplications between 500Kb and 650Kb, including the *LBX1*, *BTRC*, *POLL*, *RP11-529I10.4* (*DPCD*) and *FBXW4* genes. In one of them (C365650) *TLX1* and at least the last three exons of *FGF8* were also engaged. Thus the size of the aberrations overlapped those of previously published non-syndromic SHFM3 cases with 10q24 rearrangements. However, the affected individuals in this study had a more severe and complex clinical phenotype including specific facial gestalt (4/6 patients), hearing problems (3/6 patients) and kidney dysfunction (3/6 patients). One patient had mental retardation. The limb defects ranged from classical SHFM of hands and feet (2/6 patients) to severe symmetric terminal reduction defects of all extremities (3/6 patients). One patient (C369374) had severe reduction defects of the hands (atypical SHFM) and typical SHFM of feet. Compared to a previous survey (Elliott *et al*., 2005) the upper limbs of our patients were also more severely affected than the lower, with mainly preaxial involvement and one remaining lateral (fifth) digit ray (4/6 patients).

How does the 10q24 duplication cause syndromic and non-syndromic phenotypes

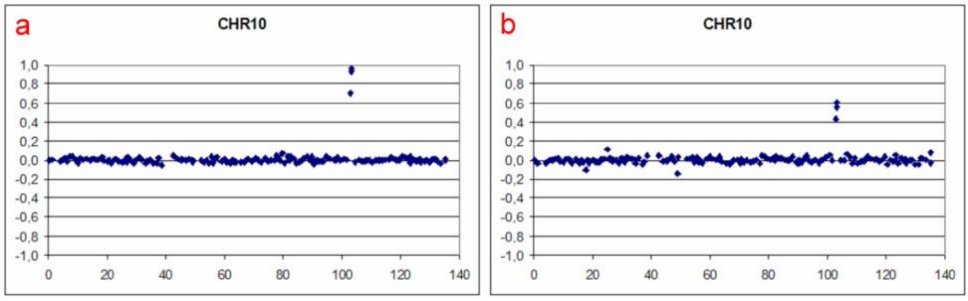
Until now, there is no explanation how the observed 10q24 genomic aberration causes the phenotype of SHFM. Expression profiling of several candidate genes did not resolve the question (Basel *et al*., 2003; Lyle *et al*., 2006). Since these experiments were done using immortalized EBV cell lines, this may not represent the expression of developmental genes during embryogenesis. In addition, *Dactylaplasia* mice were thought to be the corresponding model of SHFM3. It was shown that the semidominant phenotypes were caused by two (Dac^{1J} and Dac^{2J}) highly identical LTR retrotransposon insertions of a type D mouse endogenous provirus element (MusD).

The presence of the *Dac* phenotype was under the control of a homozygous *Mdac* trans-allele, which can completely block the development of skeletal abnormalities. However, even the observed normal skeletal phenotype in homozygous *Dac*^{2J}-animals, which are heterozygous for *Mdac*, the reduced *dactylyn* expression was not restored, thus raising questions about the causal effect of *Fbxw4*. Moreover, if the phenotype in SHFM3 patients was the result of increased copies or expression levels of gene(s) lying within the duplication, the corresponding ortholog(s) in *Dac* mice should also have a disturbed expression. None of *Lbx1, Btrc, Poll* and *Dpcd* showed any changes (Sidow *et al*., 1999; Kano *et al*., 2005, 2007). In addition, several other genes in the vicinity (*FGF8, IKKa, WNT8b, SUFU, and NFJB2*) also have a role in normal limb development. Therefore, Friedli *et al*. (2008) recently suggested that the genomic aberration could alter the proper balance and interactions between more than one cis-acting regulatory elements and their target genes. Thus, the abnormal phenotype could be the result of disturbed function of several genes and a different extent of the duplication could contribute to the observed phenotypic differences, respectively.
To answer this question, a fine mapping of the duplication size was done using a high-resolution oligo-array platform. All syndromic SHFM patients in this study, compared with non-syndromic cases, had a rearrangement encompassing the whole *FBXW4* gene at the telomeric end. This extension of the aberration cannot explain the phenotypic differences since the individual (C365650) with the largest rearrangement in this group, including at least the last three exons of *FGF8* gene, was not more severely affected. Furthermore, published cases with larger abnormalities of chromosome 10q (deletions and/or duplications), including the SHFM3 locus, do not have any SHFM like defects (Schinzel, 2001). All these data indicate that there is no obvious correlation between the size of the 10q24 duplication and the phenotype.
In one patient (C2755) a triplication of 10q24 was detected. He had almost complete reduction of all elements of both forearms and hands, and a more pronounced mental deficit, while his less severely affected sister (C224256) was a carrier of duplication (Table 1, Table 2 and Figures 1-4). Thus the presence of a cryptic triplication instead of a duplication of causative sequences within the SHFM3 locus could be another possible explanation for the observed syndromic phenotype. However, with this resolution of array-CGH platform (Agilent 244K) no similar observations for the other cases in this study were detected. Therefore the triplication itself could contribute to the phenotypic

severity in this particular patient but cannot explain the extra-skeletal abnormalities.
We can also not explain how this triplication occurred. There are no flanking low copy repeats, which could promote a recurrent non-allelic homologous recombination. Probably other DNA instability mechanisms like Break-Induced Replication Repair and Fork Stalling and Template Switching are involved (Lee *et al*., 2007; Bauters *et al*., 2008). Further research and breakpoint sequencing of more patients are necessary to unravel the genomic mechanisms causing these 10q24 rearrangements.
In 2/4 of our families, there were two affected siblings with healthy parents. Parental samples were available only from one couple (C369373 and C369374) and presence of somatic mosaicism was confirmed in the mother. Her clinical and X-ray examination did not reveal any visible abnormalities. However 3 of 21 currently known SHFM3 cases are the result of parental somatic and/or germline mosaicism for the 10q24 microduplication. Thus, at least for some patients, this also could be an explanation for the different extent of observed abnormalities. Genetic counselling of affected families should consider this.
A final possible explanation for the observed phenotypic differences could be the presence of polymorphism(s) (mutations) in causal gene(s) or regulatory motif(s) on the second allele. The skeletal abnormalities observed in our patients are similar to those observed in homozygous conditional *Fgf8* knock-out mice. The heterozygous *Fgf8* knock-outs are normal but there are other examples where a dominant phenotype in humans is only observed in homozygous mice and could be associated with long range regulator effects, e.g. *Shh* and holoprosencephaly (Chiang *et al*., 1996; Kleinjan and van Heyningen, 2005).
Additional support for a possible role of *FGF8* in the observed skeletal and extra-skeletal defects is given by transgenic studies looking for regulatory sequences downstream of *Fgf8* (Fig. 3) (Beermann *et al*., 2006; Kikuta *et al*., 2007; Visel *et al*., 2007). Intriguingly, in these transgenic animals the studied highly conserved elements can specifically drive the expression of the reporter gene within the AER, otic placode, nephrogenic cord, maxillary and mandibular arches. Some of these regulatory sequences can also partially rescue the mouse phenotype of *Fgf8* double null allele. The mutant mice survive until birth and die shortly thereafter demonstrating an abnormal facial development with maxillary and mandibular hypoplasia, an abnormal “open eye” phenotype, and an

inappropriate limb development (Beermann *et al*., 2006). This expression profile, cranio-facial and limb dysmorphism cover all the observed anomalies in our patients– maxillary hypoplasia, micrognathia, hearing problems, renal hypoplasia/dysfunction and truncation limb defects. Therefore looking for polymorphisms/ mutations in *FGF8* and its regulatory sequences in the future could extend our knowledge as to how this 10q duplication causes abnormal phenotypes.

In summary, this study demonstrates that 10q24.31q24.32 microduplications also cause syndromic forms of SHFM. There is high variability in the observed skeletal anomalies and Distal limb deficiencies with micrognathia syndrome (MIM 246560) represents the most severe end of SHFM3 spectrum. These findings strongly support the importance of array CGH analysis in entities where the underlined aetiology and/or the real extent of the clinical phenotype are not well defined. For example, Patterson-Stevenson-Fontaine syndrome (PSFS) is a condition combining partially the facial gestalt and the skeletal anomalies of DLDMS, syndromic/nonsyndromic SHFM3 patients and SHFM1 cases. The observed intra-familial variability of the PSFS is similar to the one detected in DLDMS and familial SHFM3 (Wilkie *et al*., 1997). Recently, Bigo *et al*. (2009) detected a 7q21.3 microdeletion, containing 3 genes- *DLX5*, *DLX6* and *DSS1*, in the originally described by Fontaine *et al*. (1974) family. However, no similar chromosomal aberration was found in the second PSFS family published by Patterson and Stevenson (1964). Further research needs to unravel to what extent these conditions, as well as other rare forms of acrofacial dysostoses share common aetiology and/or pathways. Until now there is no reliable explanation how the 10q24 genomic aberration causes an abnormal limb development or why some patients have extra-skeletal anomalies. It is also difficult to apply one common mechanism to explain the phenotype both in humans and *Dactylaplasia* mice. In conclusion, the real contribution of *FBXW4*, *FGF8* and other genes within or in the vicinity of the duplication associated with the observed phenotypic spectrum still remains to be discovered.



Supplemental Figure 1. 1Mb array results of chromosome 10 of patient C2755 (a) and his sister C224256 (b). Three clones, RP11-324L3, RP11-529I10 and RP11-264H19 have Log2 ratios suggestive of the presence of a triplication (a) and a duplication (b) in C2755 and C224256, respectively. X and Y axes of the graphs represent the position of the clones (in M-basepairs), and the corresponding Log2 ratios, respectively.

Genomic rearrangements of the *GREM1-FMN1* locus cause Oligosyndactyly, Radio-Ulnar synostosis, Hearing loss, Renal defects syndrome and Cenani-Lenz-like non-syndromic oligosyndactyly

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Introduction

Vertebrate limb development serves as an important model to show how different processes and pathways are synchronized and involved in morpho- and organogenesis. As limb formation is not necessary for embryonic survival, the study of several spontaneous and molecularly manipulated (transgenic) animal models contributed to the discovery of the many genes involved in limb and skeleton patterning and development. Since several of these key genes play a role in other developmental cascades, it was also believed that this knowledge could be applied to understand the pattern formation of other tissues and organs (Niswander, 2003; Tickle, 2006).

The crosstalk between two important signalling centres was found to be crucial in the complex process of limb formation - the Zone of Polarizing Activity (ZPA) and the Apical Ectodermal Ridge (AER). Key components of this mesenchymal-ectodermal (m-e) interaction are *Sonic hedgehog* (*Shh*), limb specific Fibroblast Growth Factors (FGFs), Bone Morphogenic Proteins (BMPs) and BMP antagonists like *Gremlin 1* (*Grem1*). The study of targeted mutations of limb specific *FGF* and *BMP/ BMP* antagonist genes revealed the existence of self-regulatory loops controlling the appropriate initiation and termination of m-e signalling in the coordination of limb development (Zuniga *et al*., 1999; Khokha *et al*., 2003; Scherz *et al*., 2004; Mariani *et al*., 2008; Verheyden and Sun, 2008; Benazet *et al*., 2009). In addition, the correct spatio-temporal expression of these limb morphogens depends on regulatory sequences surrounding the gene landscape in the vicinity (Zuniga *et al*., 2004; Zeller and Zuniga, 2007). A good example is the *limb deformity* (*ld*) mouse. The *ld* phenotype is autosomal recessively inherited and characterized by distinct reduction defects of the digit rays, complete radioulnar synostosis, missing fibulae and variable renal defects (Kleinebrecht *et al*., 1982). These abnormalities are most likely a result of disturbed function of *Grem1* protein in the developing limbs due to homozygous

Grem1 mutations or loss/rearrangements of a limb bud specific *Grem1* transcriptional global controlling region (GCR) within *Fmn1* gene (Zuniga *et al*., 2004; Michos *et al*., 2004; Pavel *et al*., 2007). The importance of *Grem1* in limb development is further supported by the fact that its over-expression in chick limb primordia results in truncations of distal autopod elements and syndactyly (Capdevila *et al*., 1999; Merino *et al*., 1999). Recent studies reinforce the hypothesis that a disturbed *Fmn1* protein function in limb buds may also contribute to the abnormal mouse *ld* phenotype (Zhou *et al*., 2009).

Given the recent observation that causal submicroscopic copy number variations (CNV's) can be detected in a significant proportion of patients with unexplained congenital malformations, we analyzed seventeen individuals with either non-classified syndromic congenital skeletal anomalies or severe classified limb malformations of unknown molecular aetiology.
Since the association between skeletal and renal defects is well known, the patients for array CGH analysis were selected based on the presence of combination of relatively homogenous limb and/or kidney anomalies. Two of them (Patient 1 and Patient 2) were previously published as SHFM1 (Debeer *et al*., 2004) and Cenani-Lenz syndrome (de Smet *et al*., 1992), respectively. The remaining fifteen individuals have not been assigned to a specific syndrome. All patients were examined by experienced dysmorphologists.

Clinical reports

Patient 1

The first patient (C255625) is the fourth child of healthy second cousin parents. He was born after a normal pregnancy at 41 weeks of gestation. Birth weight was 3.62kg, length 53cm and head circumference 36cm. He presented with reduction defects of the four limbs, initially classified as an atypical SHFM, bilateral subluxation of the elbows with limited pronation and supination and bilateral sensorineural hearing loss of 60 dB. Skin dimples were present on the posterior part of both elbows. His right hand had four fingers: a hypoplastic first finger with one phalanx resembling a thumb and three additional rays which could tentatively be assigned as fingers two, four and five. On the left hand there were three fingers appearing as digits two, four and five (Figure 1A,B). X-ray examination at the age of five months revealed the presence of four rays on the right hand. There was a hypoplastic first metacarpal and a thumb with only one phalanx, a delta-shaped second metacarpal, and two

Figure 1. Clinical phenotype of patients C255625 and C80286. Patient C255625 was published as an atypical case of SHFM1 (Debeer et al., 2004). There is bilateral oligosyndactyly with four fingers on the right hand (1A) and three on the left (1B). The nails of the present digits are normal. Absent second ray on both feet with remaining four toes (1C). Excretory contrast X-ray examination demonstrating agenesis of the left kidney (1D). The phenotype of patient C80286 was previously classified as an isolated Cenani-Lenz-like oligosyndactyly of all fingers of both hands. Radiographs of the hands at 6 months of age (1E) and at 27 years of age (1F) show progressive multiple fusions of metacarpal bones and phalanges with several missing skeletal elements. Consolidation of carpal bones is also present. Both feet have no skeletal anomalies (1G).

metacarpal bones with normal structure. All three fingers had three phalanges. On the left hand there were three rays, each with one metacarpal and three phalanges. The first present metacarpal was delta-shaped. The other two fingers had a normal structure. Both feet had four toes (Figure 1C). On radiographs, a missing second ray and absence of the distal two phalanges on the fifth digit were noted (Debeer *et al*., 2004). No other remarkable skeletal abnormalities were present. Abdominal ultrasound screening revealed absence of the left

kidney, which was confirmed by contrast excretory urography (Figure 1D). At the age of seven the boy had normal physical development, despite the observed skeletal anomalies. Mental development was at the lower end of the normal range. Family history was negative with regard to congenital malformations, and clinical examination of both parents revealed no limb anomalies. FISH screening for genomic deletions encompassing the SHFM1 locus on 7q21-22 as well as mutation screening of the candidate SHFM1 genes *DSS1*, *DLX5*, *DLX6* and some regulatory sequences (hI56i and hI56ii) were normal (Debeer *et al*., 2004).

Patient 2

The second patient (C80286), a boy, had a birth weight of 1500g at thirty-one weeks of gestation. Both hands were spoon-like and hypoplastic due to a complete fusion of all fingers, which was classified as a Cenani-Lenz type syndactyly (de Smet *et al*., 1992). Multiple synostoses of morphologically abnormal carpal bones were detected radiographically and several skeletal elements of the hands were absent (Figure 1E-G). Apart from two hemangiomas on the shoulders, no other abnormalities were present. His mental development was normal.

The phenotypes of the other fifteen patients are summarized in Supplemental Table 1.

Results

With a homemade 1Mb array Comparative Genomic Hybridization (CGH), two patients with a chromosomal rearrangement of the *GREM1-FMN1* locus on chromosome 15q13.3 were identified. In the first patient (Patient 1- C255625) with oligosyndactyly of the four limbs, radioulnar synostosis, hearing loss and unilateral renal aplasia (Figure 1a-d), a homozygous deletion of one BAC clone (RP11-250G15) was detected (not shown). He was previously reported as a case of Split-Hand-Foot-Malformation 1 (SHFM1) (Debeer *et al*., 2004). In the second patient (Patient 2- C80286), a two clone duplication (RP11-3D4 and RP11-250G15) encompassing the *GREM1-FMN1* locus was detected (not shown). This individual had non-syndromic Cenani-Lenz type oligosyndactyly (Figure 1E-G) (de Smet *et al*., 1992). No aberrations were detected in the remaining fifteen patients.

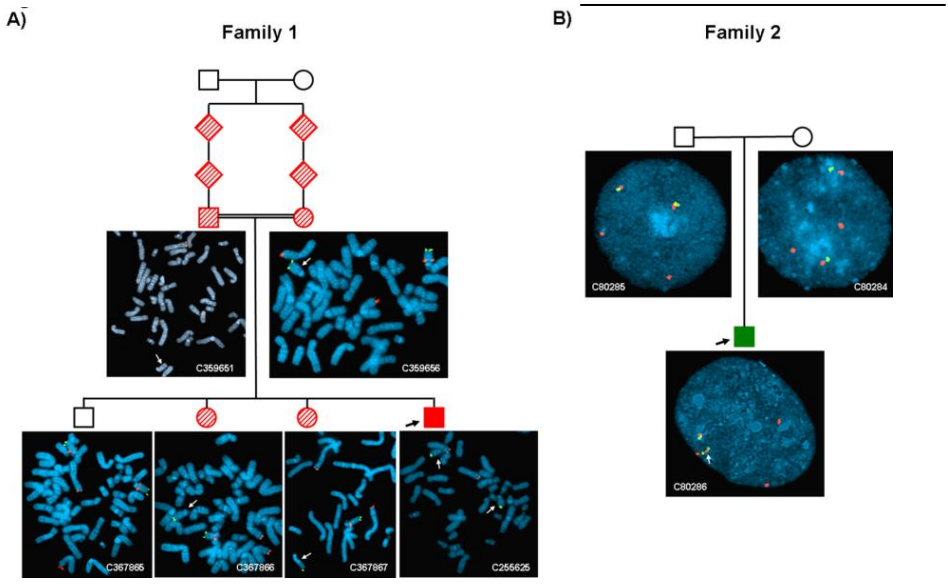

Figure 2. Pedigrees of Family 1 (case C255625) and Family 2 (case C80286). Below each individual partial metaphase spreads or interphase nuclei are shown summarizing the results of the performed FISH analysis. Filed red and green colored squares represent the patients. Striped red circles and squares show heterozygous carriers in Family 1. A) BAC RP11-250G15 (red color), with genomic position as shown on Figure 3C, was used as a FISH probe to confirm the abnormalities detected by the array CGH screening in Family 1. A chromosome 15q subtelomeric probe (green signal) served as a control. Arrows point to the aberrant alleles (with deletion) in the analyzed individuals. There was no hybridization of BAC RP11-250G15 (red signal) on both chromosomes 15 in patient C255625. This is in correlation with the array CGH results and demonstrates that the patient has a homozygous deletion of the genomic locus covered by this probe. FISH analysis showed a hybridization of BAC RP11-250G15 (red signal) on only one metaphase chromosome fifteen in heterozygous carriers of the aberration in Family 1, the parents (C359656 and C359651) and two additional siblings (C367866 and C367867) respectively. In addition, a cross-hybridization of BAC RP11-250G15 (red signal) on chromosome 3q was also observed in all analyzed metaphases. B) To confirm the presence of the microduplication in patient C80286, FISH analysis was done on interphase nuclei using two FISH probes residing within the aberration, BAC RP11-250G15 (red signal) and BAC RP11-3D4 (green signal). All patient's nuclei had one pair of a red and a green signal representing the normal allele, and a string of two red and two green signals corresponding to the allele carrying the duplication. Arrow points the aberrant allele (with duplication). The linear order of these four signals was always red-green-red-green suggesting a tandem orientation of the duplicated fragments. Both parents (C80284 and C80285) were not carriers of the aberration. In addition, all analyzed patient's and parental nuclei presented two more red signals. These two additional signals were every time separated from the others and were not paired with green one, demonstrating a cross-hybridization of BAC RP11-250G15 on chromosome 3q. Subsequently, this cross-hybridization was

also confirmed on parental and patient's chromosomal metaphase spreads (not shown).

Subsequent FISH analysis using probe RP11-250G15 (red signal) confirmed the presence of a homozygous deletion in patient C255625 (Figure 2A). Several unaffected individuals from the same family were carriers of a heterozygous deletion (Figure 2A). FISH screening with probes RP11-250G15 (red signal) and RP11-3D4 (green signal) showed the *de novo* 15q13.3 tandem duplication in patient C80286 (Figure 2B).

The micro-deletion/duplication were further delineated by high-resolution SNP array (Affymetrix 250K NspI) in patient C255625, his parents (C359651 and C359656) and case C80286. The homozygous deletion size was maximally 263kb (between SNPs SNP_A-4201883 and SNP_A-2233558) and minimally 246kb (between SNPs SNP_A-4240484 and SNP_A-2176534), encompassing the first twelve *FMN1* exons and non-coding upstream sequences (Figure 3A-C). The healthy parents (C359651 and C359656) were heterozygous carriers of the same aberration (not shown). The duplication in patient C80286 had a maximum size of 2.3Mb (between SNP_A-2022976 and SNP_A-4217332) and a minimum size of 1.7Mb (between SNP_A-2284222 and SNP_A-2248182), including both *GREM1* and *FMN1* (Figure 3A-C). Other previously undetected CNVs, that could possibly explain the phenotype of these two affected individuals, were also excluded with the SNP array.
The quality of the SNP array CGH results was validated by RT Q-PCR. Twelve primer pairs, overlapping or in the vicinity of the detected deletion/duplication breakpoints, were designed (see Materials and Methods Table 1). All results were in concordance with the SNP array CGH data (not shown). One assay was used to demonstrate the *de novo* character of the microduplication in patient C80286, (Figure 3D).
To investigate the effect of these rearrangements on *GREM1* and *FMN1* expression, we studied the gene expression in the fibroblasts of both patients (see Materials and Methods Table 1). As a highly variable expression pattern was observed in normal controls, a significant quantitative analysis was not possible (not shown). Of interest, in the patient C255625 with a homozygous deletion of the promoter and the first twelve *FMN1* exons, a low expression of the *FMN1* gene was detected (not shown).

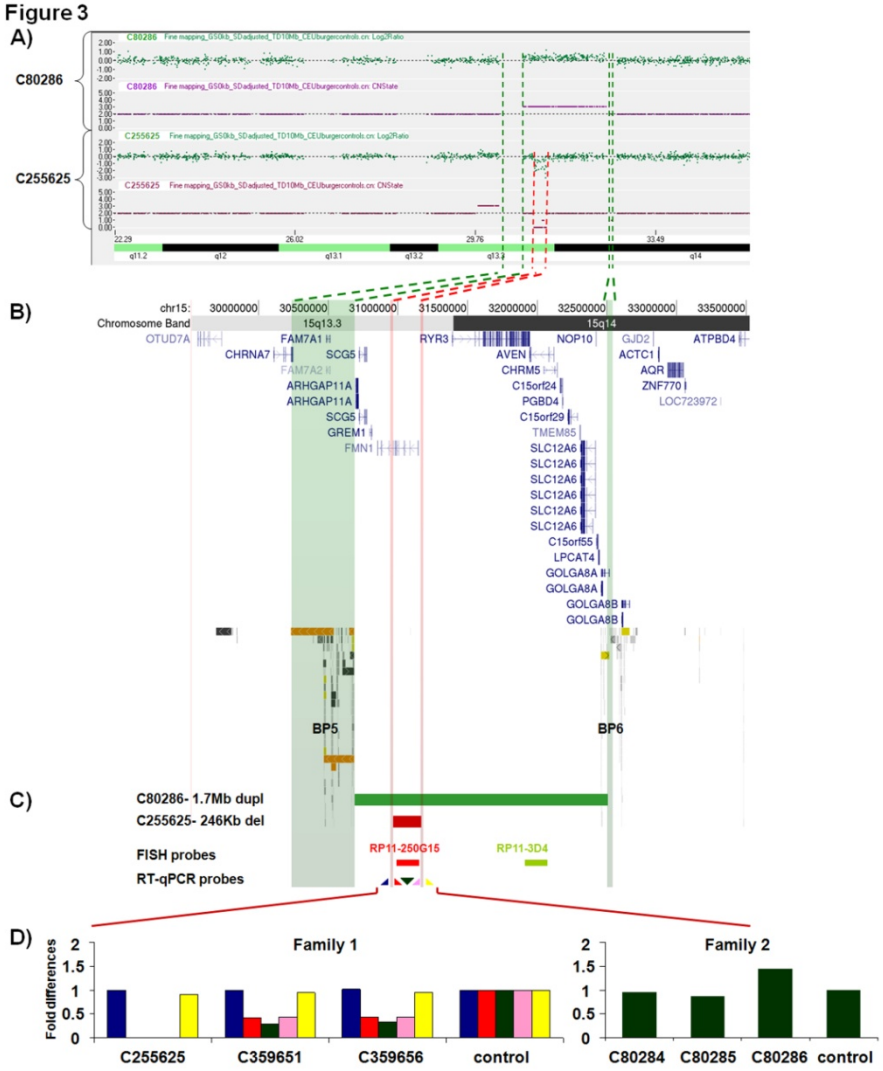

Figure 3. *GREM1-FMN1* locus on chromosome 15q13.3 and genomic positions of the detected homozygous deletion (C255625) and the *de novo* duplication (C80286) according the Human Reference Sequence March 2006, NCBI Build 36.1. A) Fine mapping of the breakpoints by 250 NspI SNP array of Affymetrix in patient C255625 and C80286. B) Genomic architecture of the aberrant locus. Transparent vertical green and pink lines demarcate the positions of the breakpoints. Low copy repeats (BP5 and BP6) flank the detected *de novo* 1.7Mb (minimal size) tandem duplication (dark green bar) of patient C80286. C) Coloured bars and triangles show the positions of the used BACs as FISH probes and some of the RT Q-PCR primers. Dark red and green bars represent the homozygous deletion (minimal size) of C255625 and the tandem *de novo* duplication (minimal size) of C80286, respectively. D) Results of the RT Q-PCR with five of the primers used to confirm the SNP array CGH results and their corresponding genomic locations according the

detected chromosomal aberrations in Family 1 and Family 2 (see Materials and methods Table 1). The bars visualize the present relative allele copy number (fold differences). Value of 1 stands for the normal diploid status, 0.5 is for hemizigousity, 0 for homozygous deletion and 1.5 for a duplication, respectively. The closest outside primers flanking the breakpoints of the detected homozygous deletion in case C255625 (Family 1) are shown in blue and yellow colours. The red and pink bars represent the closest inside primers to the breakpoints of the same patient. The green bar demonstrates the primer used for detection of the parental origin of the duplication in case C80286 (Family 2). Both parents (C80284 and C80285) have a normal diploid allele copy number.

Discussion

Identification of two new syndromes- Oligosyndactyly, Radio-Ulnar synostosis, Hearing loss, Renal defects syndrome and Cenani-Lenz-like isolated oligosyndactyly

The importance of the *GREM1-FMN1* locus in limb development has been extensively studied in animal models. We present for the first time evidence that this locus is implicated in human limb development. In one patient (Debeer *et al*., 2004) a homozygous deletion of the first twelve 5' exons of the *FMN1* gene was detected. His phenotype is highly reminiscent of the *ld* mice with oligosyndactyly, radio-ulnar synostosis and renal aplasia (Figure 1A-D). The phenotypic abnormalities in this individual are similar to those of animal models in which the function of *Grem1* was abrogated, thus resulting in an abnormal m-e interaction during limb and metanephric kidney organogenesis (Kleinbrecht *et al*., 1982; Khokha *et al*., 2003; Michos *et al*., 2004; Scherz *et al*., 2004; Zuniga *et al*., 2004; Pavel *et al*., 2007; Verheyden and Sun, 2008; Benazet *et al*., 2009). In addition he has hearing loss, which has not yet been observed in the animal models. The second patient carried a *de novo* 1.7 Mb duplication encompassing the *GREM1- FMN1* locus. His isolated Cenani-Lenz-like oligosyndactyly phenotype (Figure 1E-G) (de Smet *et al*., 1992) is similar to that of the transgenic chick in which *Grem1* over-expression causes primordial cartilage truncations and digit syndactyly due to a repression of the BMP dependant programmed cell death in the anterior necrotic zone and in the inter-digital mesenchyme. The result is a completely disorganized bone pattern of the distal autopod (Capdevila *et al*., 1999; Merino *et al*., 1999).

How the detected GREM1-FMN1 genomic rearrangements cause an abnormal phenotype

The phenotype in *ld* mice is due to a disturbed function of the *BMP* antagonist *Grem1* either by homozygous *Grem1* mutations or a loss of *Grem1* transcriptional GCR located within the neighbouring *Fmn1* gene (Figure 4) (Khokha *et al*., 2003; Zuniga *et al*., 2004; Michos *et al*., 2004; Pavel *et al*., 2007). Therefore, the coding part of *GREM1*

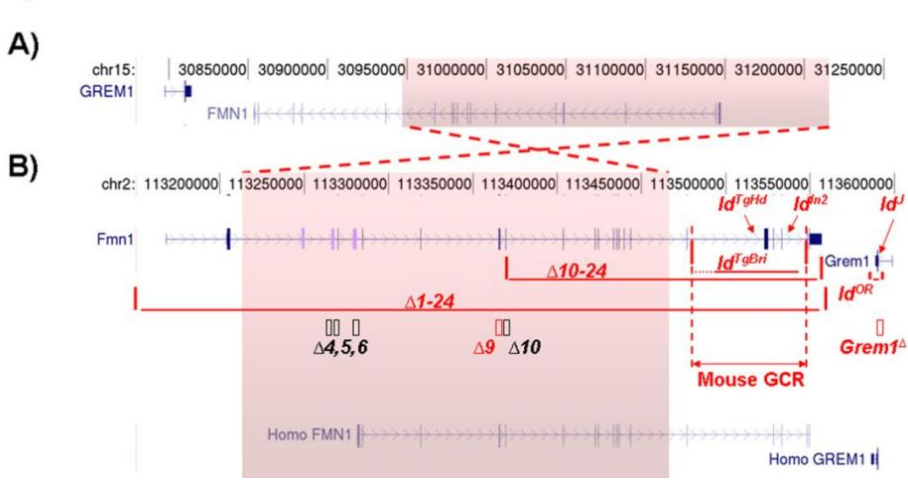

Figure 4. Alignment of the human and mouse GREM1-FMN1 locus. a) The human GREM1-FMN1 locus (Human Reference Sequence March 2006, NCBI Build 36.1) and the detected in Family 1 deletion (transparent pink bar). b) The mouse Grem1 and Fmn1 genomic structure (Zuniga et al., 2004) and position (Build 37 assembly by NCBI and the Mouse Genome Sequencing Consortium, July 2007). All known mouse mutations mapped to this landscape are indicated above or below the Grem1-Fmn1 genomic structure. Δ4, Δ5, Δ6, Δ9, Δ10-24, ldOR and Grem1Δ are targeted deletions. Point mutations (ldJ), inversions (ldIn2) and transgenic insertions (ldTgBri, ldTgHd) are also shown. Mutations marked in red cause the ld phenotype in mice (modified from Zeller and Zuniga, 2007). Transparent pink bars highlight the detected 246Kb (minimal size) deletion of patient C255625.

was directly sequenced in the fifteen patients in whom no causal CNVs were detected by the array CGH screening (Supplemental Table 1). No mutations were found. In mice, the genomic region between the nineteenth and twenty-third *Fmn1* exons contains regulatory sequences controlling the *Grem1* expression in developing limbs and kidneys (Figure 4B) (Zuniga *et al*., 2004). We determined the human homologue of this region using BLAST (blastn and bl2seq) and VISTA (genomeVISTA) alignments (Frazer *et al*., 2004;

McGinnis and Madden, 2004). The familial deletion of the first twelve *FMN1* exons observed in patient C255652 does not remove these regulatory sequences (Figure 4A,B). Therefore they were not included in the mutational analysis. Point mutations or undetected submicroscopic chromosomal aberrations of these or other limb specific *GREM1* regulatory sequences cannot be excluded in the fifteen individuals with negative mutation and CNV screening. There could also be a patient selection bias, since *ld* mice carrying *Grem1* mutations have a more severe and sub-lethal phenotype (Michos *et al*., 2004). Hence, the corresponding human phenotype could be different to those of our selected group (Kleinjan and Lettice, 2008).
In addition, the phenotype of the recently published *ld* strain is a result of a targeted deletion of *Fmn1* exon nine. This leads to a complete loss of any *Fmn1* transcript, an unexpectedly extended zone of *Grem1* expression and an increased *Bmp* activity in *ld* limb buds, indicating a direct *Fmn1* repression of the *Bmp* signalling. However the authors do not exclude the possibility that a loss of regulatory sequences could also explain the phenotype of their animal model (Zhou *et al*., 2009). Using computer analysis we searched for the presence of evolutionarily conserved sequences in the deleted genomic fragment in our patient. We found a few non-coding elements that could have a regulatory function and which were highly homologous through all tetrapods and chick but not present in xenopus, zebrafish and fugu (Figure 5) (Loots, 2008). Unfortunately, a direct proof of causal relationship between tissue specific regulatory sequences and their corresponding genes in humans is limited due to restrictions in obtaining appropriate patient samples for analysis (i.e. tissues in which the gene of interest is expressed at specific time points). All these findings make it difficult to judge to what extent either *GREM1* or *FMN1* contribute to the phenotype of the carrier of the homozygous 15q13.3 deletion in Family 1.

High-resolution SNP array analysis of the breakpoints of the second patient (C80286) showed that low copy repeats (LCR) BP5 and BP6 on chromosome 15q13.3q14.1 flank the detected duplication. Thus the aberration is proximal to the newly recognized microdeletion 15q13.3 syndrome defined between LCR blocks BP3-BP4 and BP5 (Sharp *et al*., 2008). Previous studies focused on the analysis of the 15q13.3 region did not identify a similar BP5-BP6 duplication in 3699 North American controls of predominantly European ancestry (Helbig *et al*., 2009). Analysis of 2590 individuals in our Centre by 1Mb array CGH revealed a maternally inherited benign hemizygous deletion reciprocal

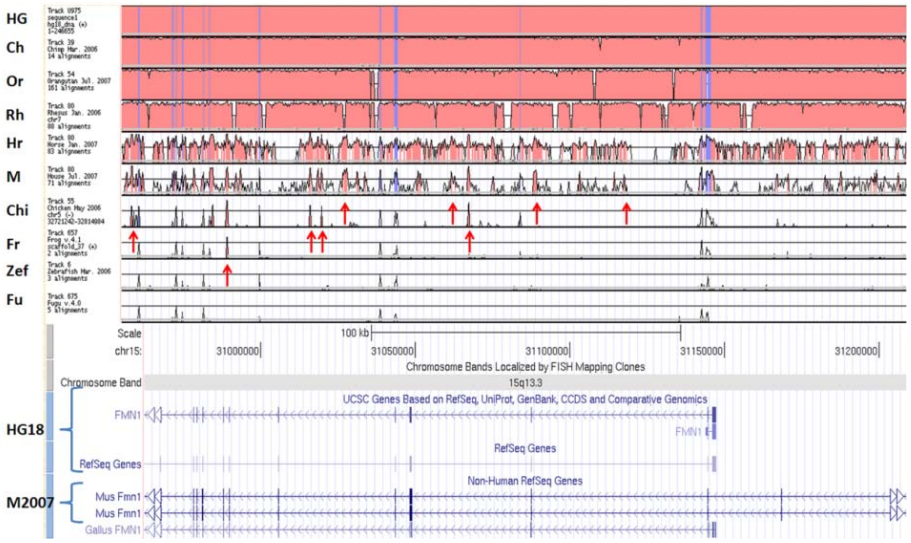

Figure 5. VISTA alignments of the deleted sequence in Patient 1 with syntenic sequences of nine other species visualized on the UCSC genome browser. HG (homo sapience), Ch (chimp), Or (orang-utan), Rh (rhesus), Hr (horse), M (mouse), Chi (chicken), Fr (frog), Zef (zebrafish) and Fu (fugu). The analysis is based on the Human Reference Sequence March 2006, NCBI Build 36.1 (HG18) and the Mouse Genome Reference Sequence 2007 (M2007). Red arrows point deeply conserved DNA stretches in tetrapods with high regulatory potential. Blue vertical lines highlight the conservation within exons and in red are labelled conserved non-coding sequences.

to the duplication in patient C80286. Hence, there is an equal proportion of detected benign hemizygous deletions and disease causing duplications for the locus between LCR BP5-BP6. None of the few published patients with larger proximal 15q13q14 deletions encompassing the BP5-BP6 locus had distal limb anomalies (Matsumura et al., 2003; Windpassinger et al., 2003; Erdogan et al., 2007).

The duplication of patient C80286 includes at least fourteen other genes besides *GREM1* and *FMN1* (Figure 3B). Currently there is no evidence in the literature that overexpression of these other genes would contribute to an abnormal limb development in humans or animal models. However, we do believe that the *de novo* character of the observed duplication in patient C80286, the absence of similar chromosomal rearrangements in a large cohort of controls screened in previous surveys (Sharp *et al*., 2008; Helbig *et al*., 2009) and the strong support of an existing corresponding animal model with similar skeletal defects (Capdevila *et al*., 1999; Merino *et al*., 1999) confirm

the causal relationship between the patient's phenotype and the increased copy number at the *GREM1-FMN1* locus. Since LCRs flank the detected aberration (Figure 3b), the most probable causative mechanism is non-allelic homologous recombination (NAHR) (Gu *et al*., 2008).

In summary, the present study delineates for the first time the human phenotype associated with a homozygous microdeletion of the *FMN1* gene. The patient's skeletal and renal defects are identical to those of *ld* mice. We also detected the human phenotype associated with a microduplication of the *GREM1-FMN1* locus in an individual with bilateral hand oligosyndactyly. No mutations of the *GREM1* gene were found among individuals with similar limb defects and/or renal anomalies. The low yield of our screening to detect genomic aberrations/point mutations in additional cases may be explained by a patient selection bias or due to restriction of the mutation analysis to the coding sequence of *GREM1*. Cenani-Lenz syndrome (CLS) was defined as a form of hand and foot syndactyly due to complete cutaneous and bony fusions. This results in disorganisation and sometimes loss of individual digit rays, and spoon-like hands. The feet are similarly affected but to a less degree. Radioulnar synostosis, mesomelic shortness, renal hypoplasia and other miscellaneous anomalies have been also observed (Harpf *et al*., 2005). No linkage to the *GREM1-FMN1* locus was found in a consanguineous family with offspring with classical spoon-like hands/ renal hypoplasia CLS (Bacchelli *et al*., 2001). Hence, even though our patients share some common clinical features (oligosyndactyly, radioulnar synostosis and renal abnormalities) with published CLS cases, they represent unique phenotypes caused by *GREM1*/*FMN1* alterations. Of course, genetic heterogeneity could be expected since abnormalities of genes participating or interacting with the SHH-GREM1-FMN1-FGF8/4 pathway in developing limbs, might cause identical/similar developmental defects. Therefore, our patients represent new clinical entities within the Cenani-Lenz phenotypic spectrum- (1) an autosomal recessive **Oligosyndactyly, Radio-Ulnar Synostosis, Hearing loss and Renal defect syndrome** due to a homozygous genomic deletion of *FMN1* gene, and (2) a dominant **Cenani-Lenz-like non-syndromic oligosyndactyly** caused by a duplication of the *GREM1-FMN1* locus. The detected tandem 15q13.3q14 duplication delineates a new candidate for recurrent genomic disorder. Analyzing more patients and further research will be necessary to unravel the clinical extent of these two new phenotypes/syndromes.

Supplemental Table 1. Summary of the mouse and patients' phenotypes

N	Mouse model	Anomalies of the forelimbs	Anomalies of the hindlimbs	Other skeletal anomalies	Extra-skeletal abnormalities/ others
1	*ld* mice	oligosyndactyly (reduced number of digits; It is difficult to assign the remaining rays with certainty to a specific ray number), fusions between metacarpal bones, radioulnar synostosis	oligosyndactyly (reduced number of digits; It is difficult to assign the remaining rays with certainty to a specific ray number), metatarsal/tarsal fusions, fibular aplasia	none	usually unilateral kidney aplasia (the frequency of occurrence depends on the background)
2	homozygous *Grem1*$^{\Delta ORF}$ mice	oligosyndactyly (reduced number of digits; It is difficult to assign the remaining rays with certainty to a specific ray number), fusions between metacarpal bones, radioulnar synostosis	oligosyndactyly (reduced number of digits; It is difficult to assign the remaining with certainty to a specific ray number), metatarsal/tarsal fusions, fibular aplasia	none	bilateral aplasia of kidneys and ureters, lung septation defects, die shortly after birth
N	**Patients**	**Anomalies of the Upper limbs**	**Anomalies of the Lower limbs**	**Other skeletal anomalies**	**Extra-skeletal abnormalities/ others**
1	C255625	oligosyndactyly , subluxation of the elbow, radioulnar synostosis	oligodactyly	none	hearing loss, left renal aplasia, borderline mental development
2	C80286	spoon-like hands, fusions of several metacarpal/carpal bones, disorganization of digit rays	none	none	none
3	C316387	radioulnar synostosis	none	none	renal hypoplasia
4	C333667	radioulnar synostosis	none	none	ectopic kidney, learning problems, micrognathia
5	C270528	proximal symphalangism, radioulnar synostosis	none	none	familial (daughter with radioulnar synostosis, oral cleft and deafness)

6	C176689	radioulnar synostosis	none	none	oral cleft, bilateral renal anomalies
7	C114258	radioulnar synostosis	none	none	mental retardation, facial dysmorphism
8	C158557	none	metatarsal/tarsal synostosis	none	mental retardation, facial dysmorphism
9	C274140	radioulnar synostosis	none	none	facial dysmorphism
10	C151197	carpal synostosis	none	none	mental retardation
11	C145176	proximal symphalangism	none	vertebral fusions (C2-C4)	none
12	C342504	hypoplastic middle phalanges V, radioulnar synostosis	none	none	heterochromia of the iris
13	C67651	left hand oligosyndactyly with disorganization of digit rays; absent hand bones, single hypoplastic forearm bone with elbow soft tissue webbing on the right side	none	none	congenital heart defect (VSD), micrognathia, congenital anosmia
14	C353684	soft tissue syndactyly, mesoaxial polydactyly, fusion of proximal phalanges	bilaterally short third metatarsal and absent phalanges of digit III	none	familial
15	C368079	left hand oligosyndactyly with disorganization of digit rays; absent hand bones and single rudimentary forearm bone on the right side	oligosyndactyly with metatarsal/tarsal fusions, disorganization of digit rays	none	uterus bicornis
16	C369368*	Cenani-Lenz syndactyly	NA	NA	NA
17	C186185	hypoplastic thumbs, metacarpals I and radius, radiohumeral synostosis	none	none	dysplastic ears

*- This patient was referred with a diagnosis of Cenani-Lenz syndrome. No detailed clinical description is available; NA- not available.

2q31 microdeletion syndrome- redefining the associated clinical phenotype

Adapted from 2010 (manuscript submitted)

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Introduction

Several patients with chromosome 2q31 interstitial deletions have been described. The clinical phenotype was first delineated by Boles *et al*. (1995) and consists of developmental delay, facial dysmorphism, a variety of limb defects and other internal organ anomalies affecting the brain, eyes, heart, and the uro-genital system (Boles *et al*., 1995; Maas *et al*., 2000). Remarkably, the spectrum of upper/ lower limb abnormalities can range from (1) ectrodactyly/ monodactyly, through (2) syndactyly and (3) brachydactyly to (4) isolated camptodactyly or (5) clinodactyly. The *HOXD* cluster was initially accepted as a major candidate responsible for the observed abnormal limb morphogenesis (Moller *et al*., 1984; Boles *et al*., 1995). Genes important for the mental, cranio-facial and heart development were thought to be located more proximally on chromosome 2q24 (Maas *et al*., 2000).

Recently, based on the phenotype of patients with overlapping interstitial deletions of the 2q31 region, a new locus responsible for split foot-hand malformation (SHFM) was proposed- SHFM5. It was positioned proximally to the *HOXD* cluster between *EVX2* and marker D2S294 thus including *DLX1* and *DLX2*. The last two genes have been suggested as the most probable candidates causing the phenotype (Goodman *et al*., 2002).

In an attempt to investigate further the phenotype/ genotype correlations at the 2q31 region and to refine the hypothetical SHFM5 locus, five patients with different size chromosome deletions encompassing this band were collected for fine breakpoint mapping with array CGH. By alignment of the detected aberrations and reviewing previously published 2q31 deletion cases (Nixon *et al*., 1997; Del Campo *et al*., 1999; Slavotinek *et al*., 1999; Goodman *et al*., 2002; Pereira *et al*., 2004; Bijlsma *et al*., 2005; Langer *et al*., 2006; Mencarelli *et al*., 2007; Svensson *et al*., 2007; Pescucci *et al*., 2007; Monfort *et al*., 2008; Prontera *et al*., 2009; Tsai *et al*., 2009) we were able to show that hemizygosity of only the *HOXD* genes and *HOXD* regulatory sequences in vicinity (Spitz *et al*., 2003; Gonzalez *et al*., 2007) is responsible for the observed limb defects in these

individuals. Even more, for the first time we demonstrate that the 2q31.1 microdeletion is a clinically recognizable contiguous gene syndrome, consisting of two partially overlapping but distinct loci responsible for the specific facial gestalt and skeletal abnormalities.

Clinical reports

All five patients have been examined by at least one of the authors and long term follow-up was available for all individuals.

Patient 1

This boy is the first child of healthy and young Caucasian parents. Birth weight was 3.500kg (P25-P50) and the length 49cm (P3-P10). At the age of 4 and 1/2 years he had short stature (<P3) and moderate to severe mental retardation, no speech, generalised muscular hypotonia, relative microcephaly (P3-P10), thin, sparse hair, a prominent forehead, ptosis, down-slanting palpebral fissures, a bulbous nasal tip, cleft soft palate, open mouth with downturned corners, everted and thick lower lip, low-set ears with large lobes, short neck with low-set hair line, camptodactyly, bilateral fifth finger clinodactyly, and bilateral syndactyly of toes II-V. In addition, a ventricular septal defect closed spontaneously (Figure 1A-D).
MRI imaging revealed a complex brain anomaly with hydrocephaly, hypoplastic corpus callosum, small sella tursica with hypoplastic pituitary gland and an ectopic neurohypophysis (Figure 1E).
Radiographs of hands and feet showed delayed bone age, relatively elongated proximal and hypoplastic middle phalanges particularly of the second and fifth fingers, irregular ossification of tarsal bones, wide metatarsals with irregular metaphyses and epiphyses, widening of the first toe rays with very short first proximal phalanges, and absent middle phalanges of toes II-V (Figure 1F,G).
High resolution karyotyping revealed a *de novo* interstitial deletion 2q24.3q31.

Patient 2

The proposita is the second child of healthy parents. After a difficult pregnancy complicated with bleeding in the 12th week, she was born by Caesarean section at the 36th week of gestation because of transverse position of the foetus. There were signs of prematurity. Her birth weight was 1.900kg (P3-P10), length 41cm (<P3) and head circumference 29cm (<P3). Due to adaptation problems she was admitted to the neonatal intensive care unit for a short period. The

Figure 1. Patients phenotypes. A-G) Facial gestalt, complex CNS anomaly and limb defects of Patient 1. There is hypoplasia of the second and fifth phalanges (F), broad first toe ray, wide and short, bullet-like first proximal phalanges of both feet and absent middle toe phalanges II-V (G). H-K) Patient 2 at three months (H) and two years of age (I). She presents bilateral toe syndactyly II-IV (J,K), broad first metatarsals and first toe phalanges, wide and short (bullet-like) first proximal phalanges (L), absent ossification centres of middle phalanges II-V (L). Brain imaging shows cortical CNS abnormalities and bilateral periventricular cysts (M). N-P) Patient 3 has elongated and asymmetric face with long nose, and high nasal bridge (N). Mild syndactyly III-IV of hands (O) and bilateral hallux valgus, sandal gap, and short 5th metatarsal are present (P). Q-R) Patient 4 at one year and four

years, respectively (see the clinical report). S-T) Facies of patient 5 (see the text for more details).

mother had one previous miscarriage and a normal older daughter from another relation. Patient 2 was referred to the Genetic Clinic because of developmental delay, microcephaly (<P3) and facial dysmorphism characterised by a narrow forehead with a prominent metopic suture resulting in a trigonocephalic shape of the head, downslanting palpebral fissures, hypotelorism, shallow orbits with protruding eyes, ptosis, asymmetric pupils (R>L), bulbous nasal tip with a small pit, protruding ears with simple pinnae, a thin upper lip, downturned corners of the mouth and mild micrognathia (Figure 1H,I). The left foot had complete cutaneous syndactyly II-V. On the right foot there was only soft tissue-syndactyly of the second and third toes. Both halluces looked larger and were separated by a wide gap from the second digit ("sandal gap" sign) (Figure 1J,K). In addition, there were also two flat pre-sacral haemangiomata, dimples on both elbows, and a ventriculo-septal heart defect that closed spontaneously. At two years of age her height was below the third percentile (<P3) and she had thin hair. There was marked microcephaly (<P3) and developmental delay with a developmental age of seven months. A conventional G-banded karyotype was normal.
Babygram and Radiographs of lower limbs showed short second and fifth middle phalanges of the hands, wide first toe rays, short and broad first metatarsals, short, bullet-like middle phalanx of the first toes and absent ossification centres of II-V digits of the feet (Figure 1L). Brain imaging revealed abnormal cortical gyration and periventricular cyst lesions affecting the thalami (Figure 1M).

Patient 3

The third patient is the second child of healthy and young Caucasian parents. She was born at 37 gestation weeks with a birth weight of 3.000kg (P50), length of 49cm (P25-P50) and head circumference of 34cm (P25-P50). Due to severe neonatal hypotonia, feeding problems and an increased risk for Sudden Infant Death Syndrome she was on monitoring until the age of four months. Over the years she developed a progressive thoraco-lumbar scoliosis with short stature, persistent hypotonia, hyperlaxity and tapering fingers. At 13½ years clinical re-evaluation revealed a standing height at the 3rd centile, truncal hypotonia, a prominent thoracic kyphoscoliosis, and elongated asymmetric face with long nose, high nasal bridge, full lower lip and retrognathia (Figure 1N). There was developmental delay and marked

microcephaly (<P3). The hands had tapering fingers, mild syndactyly of digits III-IV and smooth palmar creases (Figure 1O). Short fifth metatarsals, increased distance between the first and second toes with hallux valgus and flat foot arches were present bilaterally (Figure 1P). In addition she suffered from urinary infections and a urinary reflux was detected. A G-banded karyotype was normal and *FMR-1* gene mutations were excluded.

Patient 4

Patient 4 is the eldest daughter of two children of non-consanguineous parents. Her brother is healthy. She has moderate intellectual disability with slow developmental milestones and is always good-humoured and continually active. She is unable to concentrate and therefore attends special education.

At 6 years of age her height was 106.5cm (P3), weight 17kg and head circumference 48cm (<P3). She is thus microcephalic and displays downslanting palpebral fissures, epicanthus inversus, a fine nose bridge and a pointed chin (Figure 1Q,R). Her teeth are tightly packed. She has clinodactyly of the fifth fingers and foetal finger pads.

No chromosomal abnormalities were detected by conventional karyotyping.

Patient 5

This girl is the second child of healthy, non-related parents. She was born at term with normal physical parameters after an uncomplicated pregnancy. At the age of 6 months, she was admitted to the hospital because of febrile seizures and anti-epileptic therapy was initiated. Clinical examination at the age of 9 months revealed developmental delay with pronounced hypotonia. She had a narrow thorax, a narrow forehead with a prominent metopic suture, a short nose with thin nares, depressed and wide nasal bridge, a long and smooth philtrum, blepharophimosis, epicanthic folds, nystagmus, strabismus, Duane anomaly, simple ears with uplifted ear lobules, a high arched palate, a small tent-shaped mouth, micrognathia, and relatively small hands and feet (Figure 1S,T). Both the head circumference and length were at the 25th centile (P25). High resolution karyotyping did not reveal any abnormalities.

Results

Identification of a 2q13 deletion in the five patients

High resolution standard karyotyping revealed a 2q31 chromosomal aberration in Patient 1. Subsequently, a 1Mb array CGH analysis

confirmed this and excluded the presence of other submicroscopic copy number variations that would contribute in addition to the phenotype of this individual. The 2q31 microdeletions of Patients 2-5 were detected by routine 1Mb array CGH screening. In all five individuals, the detected chromosomal abnormalities were *de novo*.

Fine mapping of the size of the detected 2q31 deletions

To confirm the 1Mb array CGH data and to define the correct size of the deleted segment, a chromosome 2 tiling path array CGH analysis was carried out. All results were consistent with those of the 1Mb array CGH screening.

An 11.36Mb interstitial deletion flanked by BACs RP11-656M07 and RP11-600I19 was detected in Patient 1. This aberration removes 134 genes including *DLX1*, *DLX2* and the entire *HOXD* cluster on chromosome 2q31.1 and extends from band 2q31.1 to band 2q31.3 (Figure 2A).

Patient 2 carried the largest deletion, which removes 16.9Mb on chromosome 2q31.1-q32.1 from BACRP11-703L16 to BAC RP1189E07, thus encompassing 140 genes. The *HOXD* and *DLX* were present in a hemizygous state (Figure 2A).

Patient 3 had a complex karyotype. A 2.74Mb deletion on chromosome 2q31.1-q31.2 (between BACs RP11-118L08 and RP11-592D06) was found. The detected aberration starts 59Kb proximal to the *EVX2* gene and extends to the *TNN* gene (Figure 2A). There was an additional deletion of chromosome 16p13.11 with a minimum size of 1.9Mb (between BACs RP11-489O1 and RP11-288I13) and maximum size of 7Mb (between BACs RP11-174B4 and RP11-489A11). This aberration is identical to the recently described microdeletions in this locus, which are a predisposition factor for developmental disabilities (Hannes *et al*., 2009). The reciprocal duplications are likely benign polymorphisms. No consistent limb abnormalities were found in individuals carrying either 16p13.11 deletions or duplications (Hannes *et al*., 2009). FISH analysis with BAC RP11-49401 (chromosome 16p13.11) and BAC RP11-157E8 (chromosome 2q31.1) labelled with Spectrum Orange™-dUTP was used to confirm the 1Mb array CGH results (data not shown).

In Patients 4 and 5, a 6.32Mb deletion on chromosome 2q24.3-q31.1 (between BACs RP11-760C21 and RP11-1E20) and a 4.12Mb deletion on chromosome 2q31.1 (between BACs RP11-121K19 and RP11-388J7) were found, respectively. In both patients the detected aberration removed *DLX1* and *DLX2* genes but the *HOXD* cluster remained intact (Figure 2A).

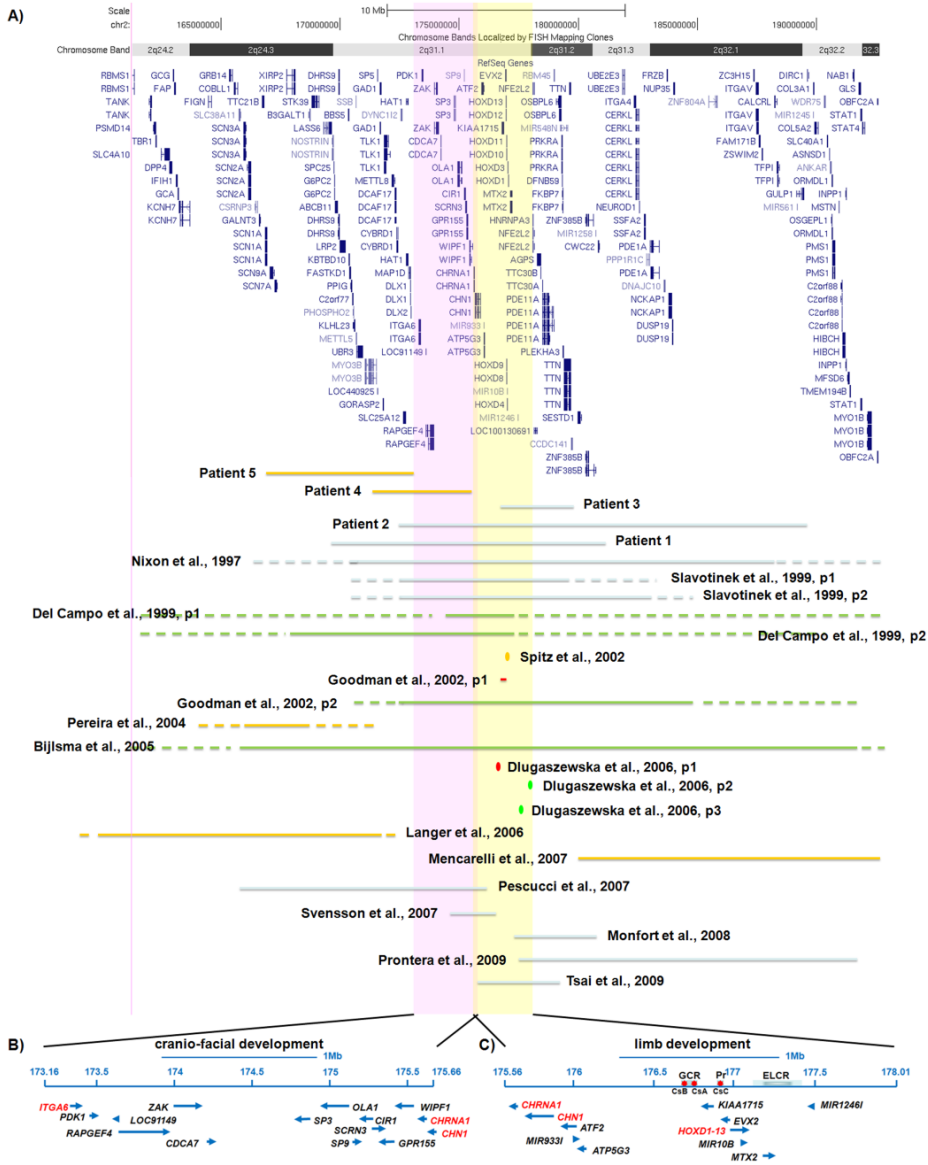

Figure 2. Chromosome 2q24.2-q32.3. A) Alignment of the 2q deletions of patients 1-5 and literature cases (Nixon *et al.*, 1997; Del Campo *et al.*, 1999; Slavotinek *et al.*, 1999; Goodman *et al.*, 2002; Pereira *et al.*, 2004; Bijlsma *et al.*, 2005; Langer *et al.*, 2006; Mencarelli *et al.*, 2007; Svensson *et al.*, 2007; Pescucci *et al.*, 2007; Monfort *et al.*, 2008; Prontera *et al.*, 2009; Tsai *et al.*, 2009). Yellow lines mark patients without skeletal defects. Blue and green lines show patients with "typical", and severe limb defects (SHFM/ monodactylous ectrodactyly), respectively. The red line represents a patient with synpolydactyly (SPD) (Goodman *et al.*, 2002). Red,

green and orange ovals point to the 2q31 breakpoints of patients carrying apparently balanced chromosomal translocations and an associated abnormal phenotype: mesomelic skeletal dysplasia (orange) (Spitz *et al*., 2002), SPD (red) (Dlugaszewska *et al*., 2006), and SHFM/ monodactylous ectrodactyly (green) (Dlugaszewska *et al*., 2006), respectively. Transparent pink (facies) and yellow (limbs) vertical bars demarcate the FG and LA specific loci. Both regions with the candidate genes for cranio-facial and limb development are highlighted in sections (B) and (C). Genes associated with OMIM diseases are highlighted in red. In section (C) blue bars and red stars show the positions of *HOXD* regulatory sequences in mice as follow: GCR is for global control region, Pr for Prox, ELCR for early limb control region, and CsA, CsB and CsB for control sequence A, B, and C, respectively (Spitz *et al*., 2003; Zakany *et al*., 2004; Gonzalez *et al*., 2007).

Delineation of a common locus for Limb Anomalies

In two out of five patients in this study (Patients 1 and 2) there were mild limb defects associated with a deletion encompassing the *HOXD*, *DLX1* and *DLX2* genes. Thus the detected aberrations remove the entire SHFM5 locus proposed by Goodman *et al*. (2002). In one additional case with mild abnormalities of hands and feet (Patient 3) there was a 2q31.1 deletion affecting only the *HOXD* cluster, but not *DLX1* and *DLX2*. No limb defects were present in the remaining two individuals (Patients 4 and 5). However they were carriers of a more proximal 2q31.1 deletion including *DLX1* and *DLX2* genes, but not the *HOXD* locus. In addition, we reviewed other published cases in the literature for which any molecular data for the size of the detected 2q31.1 aberration were available (Nixon *et al*., 1997; Del Campo *et al*., 1999; Slavotinek *et al*., 1999; Goodman *et al*., 2002; Pereira *et al*., 2004; Bijlsma *et al*., 2005; Langer *et al*., 2006; Mencarelli *et al*., 2007; Svensson *et al*., 2007; Pescucci *et al*., 2007; Monfort *et al*., 2008; Prontera *et al*., 2009; Tsai *et al*., 2009).

Alignment of the detected 2q31 aberrations in our patients with those from the literature clearly demonstrates that only the *HOXD* cluster and surrounding up-/ down-stream sequences are responsible for the observed Limb Anomalies (LA) in individuals with 2q31 microdeletion syndrome (Figure 2A). This critical LA region extends 1.5Mb centromeric and 1Mb telomeric from the *HOXD* genes. It covers approximately 2.5Mb including all well defined *HOXD* regulatory elements such as the Global Control Region (GCR) and Proxy (Pr) with the Control sequences A (CsA), B (CsB), and C (CsC), as well as the hypothetical Earlier Limb Control Region (ELCR) (Figure 2C) (Spitz *et al*., 2003; Zakany *et al*., 2004; Gonzalez *et al*., 2007; Yamagishi *et al*., 2007). Of interest, in four published patients with 2q31.1 microdeletions and limb defects (Svensson *et al*.,

2007; Pescucci *et al*., 2007; Monfort *et al*., 2008; Prontera *et al*., 2009), the detected chromosomal aberration did not remove any of these currently known limb specific *HOXD* enhancers/ suppressors. Therefore, based on these findings and some preliminary data from animal models (Spitz *et al*., 2003; Zakany *et al*., 2004; Gonzalez *et al*., 2007; Spitz- personal communications), the presence of more regulatory elements involved in the limb development between the GCR and *ATP5G3* gene, and downstream to the *HOXD* cluster is hypothesized (Figure 2C). Indeed, there are several highly conserved sequences in tetrapods within this region and further research should unravel their importance for autopod development (not shown). In addition to the *HOXD* group, this specific LA locus harbours ten other genes. Two of them are associated with OMIM diseases (*CHRNA1* and *CHN1*). However, patients with 2q31 microdeletions do not share common features with any of these OMIM phenotypes.

Delineation of a common locus for the Facial Gestalt

Three patients in this study had similar facial features (Figure 1A,B,H,I,Q,R- Patients 1, 2 and 4). A genotype/phenotype correlation, based on their phenotypes and cases in the literature, reveals a common 2.4Mb locus (maximum size) on chromosome 2q31.1 that could harbour a causal gene(s) for the observed Facial Gestalt (FG) (Figure 2A,B). This critical FG region covers at least 15 known genes. Mutations in three of them are implicated in known OMIM disorders. Some of these "candidates" are transcription factors or are involved in the cell division cycle. Hence, they might be functionally important for the cranio-facial development. Of course, a positional effect of the detected 2q31 aberrations upon the function of genes in vicinity like *DLX1* and *DLX2* cannot be excluded since a partial facial phenotype could be observed in patients with 2q31 deletions ending in a close proximity to the defined critical FG locus in our study.

Discussion

Five individuals were analyzed by array CGH in this study (Table 1). They were carriers of partially overlapping 2q31 deletions extending either centromerically or telomerically. Subsequently, their data from the molecular karyotyping were aligned with those of published patients (Nixon *et al*., 1997; Del Campo *et al*., 1999; Slavotinek *et al*., 1999; Goodman *et al*., 2002; Pereira *et al*., 2004; Bijlsma *et al*., 2005; Langer *et al*., 2006; Mencarelli *et al*., 2007; Svensson *et al*., 2007; Pescucci *et al*., 2007; Monfort *et al*., 2008; Prontera *et al*., 2009; Tsai *et al*., 2009). All analyzed individuals had different sized 2q31

aberrations. No common breakpoints were observed. Therefore, a mutual genomic mechanism cannot be suggested with certainty for the occurrence of these deletions (Figure 2A) (Gu *et al*., 2008). Specific loci for the limb defects (LA) and for the facial gestalt (FG), both located on chromosome 2q31.1, were found. Based on these results, we suggest that the detected common 2q31.1 microdeletion is a recognizable contiguous gene syndrome. (Figure 2A-C). The phenotype is characterized by moderate to severe mental retardation, microcephaly, short stature, hypotonia, specific facial dysmorphism and variable limb defects with distinct pattern. The common facial gestalt includes a narrow forehead sometimes with prominent metopic suture, craniosynostosis (very rare), a small nose with bulbous tip, long and smooth philtrum, downslanting palpebral fissures, thin upper lip, thick and everted lower lip, low set and dysplastic ears, and micrognathia. The observed upper limb defects are clenched hands, shortening of middle phalanges leading often to clinodactyly of the fifth finger, brachy-metacarpy and partial cutaneous syndactyly. Absent middle and terminal phalanges, sometimes associated with nail hypoplasia, partial to complete cutaneous syndactyly II-IV, short metatarsals and wide first ray tubular bones are typical for the feet. Due to a digit hypoplasia and syndactyly, there could be a wide distance between the hallux and the remaining toes. This has been occasionally described as an ectrodactyly (Boles *et al*., 1995; Maas *et al*., 2000). Some affected individuals have fusions of phalanges, metatarsals and metacarpals. There is tendency for the lower limbs to be more often and more severely affected than the upper limbs (Table 1). Congenital defects of the heart, brain and eyes, as well as clefts, scoliosis, an abnormal vertebral segmentation and seizures are common but not specific. The presence and extent of these additional anomalies clearly depends on the size and the direction of the detected 2q31 deletion (Table 1 and Figure 2A).

Limb development and limb defects

A 2.5Mb critical locus (LA) for the observed limb anomalies in patients with 2q31.1 deletion syndrome was delineated including the *HOXD* genes and its regulatory sequences (Figure 2C). A *HOXD* haploinsufficiency is the only plausible explanation for the disturbed limb development in these individuals since their hand and foot defects are comparable with those of *HOXD* mutant mouse models (Zakany and Duboule, 1996; Kmita *et al*., 2002; Zakany *et al*., 2004).
There were also a few published “atypical” cases that had more severe

Table 1. Clinical features of the selected patients with molecularly confirmed deletions encompassing chromosome 2q31

Patients	Sex	General findings	Cranio-facial
Patient 1 (this study)	**M**	**growth retardation, short stature, developmental delay, hypotonia**	**microcephaly, bitemporal narrowing, bulbous nasal tip, flat long philtrum, thin upper lip, thick/ everted lower lip, cleft palate, micrognathia, low-set dysplastic ears**
Patient 2 (this study)	**F**	**low birth weight, growth retardation, short stature, developmental delay, hypotonia, pre-sacral haemangioma, dimples on both elbows**	**trigono-microcephaly, bulbous nasal tip, flat long philtrum, thin upper lip, everted lower lip, cleft palate, micrognathia, protruding dysplastic ears**
Patient 3 (this study)	**F**	**growth retardation, short stature, developmental delay, hypotonia, kyphoscoliosis**	**microcephaly, long face, facial asymmetry, high nasal bridge, thick lower lip, retrognathia**
Patient 4 (this study)	**F**	**developmental delay, short stature**	**microcephaly, thin nose with bulbous tip, smooth philtrum, thin upper lip, small pointed chin**
Patient 5 (this study)	**F**	**developmental delay, hypotonia, seizures**	**trigonocephaly, short nose, smooth philtrum, small mouth, low-set dys-plastic ears micrognathia,**
Nixon *et al*., 1997	**M**	**low birth weight , growth retardation, short stature, developmental delay**	**microcephaly, cranio-synostosis, bitemporal narrowing, bulbous nasal tip, flat long philtrum, thin upper lip, high palate, micrognathia, low-set ears**

Eyes	Upper limbs	Lower limbs	Others
ptosis, downslanting palpebral fissures	camptodactyly, clinodactyly V, delayed bone age, brachidactyly, hypoplastic middle phalanges II and V	syndactyly II-V, sandal gap, irregular ossification, wide metatarsals, short and wide (bullet-like) proximal phalanx I, absent middle phalanges II-V	spontaneously closed VSD, hydrocephaly, hypoplastic corpus callosum, small sella turcica, hypoplastic pituitary gland, ectopic neurohypophysis
ptosis, downslanting palpebral fissures, hypotelorism, shallow orbits, protruding eye bulbs, asymmetric pupils	camptodactyly, clinodactyly V	left syndactyly II-V, right syndactyly II-III, sandal gap, broad first rays, bullet-like proximal phalanx I, absent middle phalanges II-V	spontaneously closed VSD, cortical brain abnormalities, bilateral periventricular cysts
no abnormalities	tapering fingers, syndactyly III-IV, smooth palmar creases	sandal gap, medial deviation of the halluces, short metatarsal V	urinary reflux
epicanthus inversus, downslanting palpebral fissures	clinodactyly V, foetal finger pads	no abnormalities	no abnormalities
blepharophimosis, epicanthic folds, strabismus, nystagmus, Duane anomaly	no abnormalities	no abnormalities	no abnormalities
proptosis, hypertelorism, small and downslanting palpebral fissures, coloboma of iris, retina and optic nerve	camptodactyly, clinodactyly V, single palmar creases	left syndactyly II-V, right syndactyly III-V, sandal gap, joint contractures	ASD, VSD, PDA, bicuspid pulmonary valve, bilateral cryptorchidism and inguinal hernia

Table 1 continued

Patients	Sex	General findings	Cranio-facial
Slavotinek *et al*., 1999, p1	**F**	**low birth weight, growth retardation, short stature, developmental delay, scoliosis, seizures**	**microcephaly, facial asymmetry, bitemporal narrowing, short nose with bulbous tip, long philtrum, cleft uvula, micrognathia, simple ears**
Slavotinek *et al*., 1999, p2	**M**	**low birth weight, growth retardation, short stature, developmental delay, hypotonia, seizures,**	**trigono-microcephaly, bulbous nose tip, flat long philtrum, thin upper lip, micrognathia, low-set ears**
Del Campo *et al*., 1999, p1	**M**	**growth retardation, developmental delay**	**Microcephaly**
Del Campo *et al*., 1999, p2	**M**	**growth retardation, neonatal death**	**cleft palate, low-set ears**
Goodman *et al*., 2002, p1	**F**	**normal**	**no abnormalities**
Goodman *et al*., 2002, p2	**F**	**growth retardation, developmental delay, seizures**	**preauricular tag**
Pereira *et al*., 2004	**F**	**low birth weight , growth retardation, seizures, developmental delay**	**microcephaly, dysplastic ears**

Eyes	Upper limbs	Lower limbs	Others
ptosis, small and downslanting palpebral fissures	camptodactyly, tapering fingers,proximaly placed thumbs, clinodactyly V, single palmar creases	syndactyly II-III, sandal gap, short metatarsal V	dilatation of brain ventricles, hypoplastic external genitalia, anteriorly placed anus, hiatus hernia
ptosis, downslanting palpebral fissures	camptodactyly, tapering fingers, clinodactyly V, single palmar creases	left syndactyly II-III and IV-V, right syndactyly II-V, short metatarsal V	bilateral cryptorchidism, cerebral atrophy, dilatation of brain ventricles
NA	thin humerus, single radius-like forearm bone, single metacarpal, one biphalangeal digit	bowed femur, absent tibia and fibula, two tarsal bones, one metatarsal, one biphalangeal digit	cryptorchidism, small phallus, hypospadia
hypertelorism, left microphthalmia, left optic nerve hypoplasia	left single radius-like forearm bone, right short radius and ulna, monodactyly	several not well defined tarsal bones, two digits	ASD, arrhinencephaly, ectopic brain gray matter, multicystic hypoplastic kidney, penoscrotal transposition, micropenis
downslanting and small palpebral fissures	SPD, clinodactyly V	broad halluces, syndactyly II-V, absent middle phalanges II-V, short metatarsals II-V, partial metatarsal II duplication	no other abnormalities
blepharophimosis, retinal coloboma	camptodactyly, clinodactyly V	classical SHFM with present two digits	NA
downslanting and small palpebral fissures, dysplastic ears	single palmar crease	syndactyly II-III	VSD

Table 1 continued

Patients	Sex	General findings	Cranio-facial
Bijlsma *et al*., 2005	**F**	**17g.w. foetus, pregnancy termination**	**high nasal bridge, long philtrum, low-set ears, micrognathia**
Langer *et al*., 2006	**F**	**low birth weight , growth retardation, developmental delay, hypotonia, seizures**	**micrpcephaly, prominent metopic suture, dysplastic ears, micrognathia**
Mencarelli *et al*., 2007	**M**	**low birth weight, developmental delay, kyphoscoliosis**	**coarce face, high forehead, synophrys, bifid nasal tip, micrognathia, dysplastic ears**
Pescucci *et al*., 2007	**F**	**low birth weight, growth retardation, short stature, developmental delay, seizures**	**microcephaly, cleft palate, high nasal bridge, bulbous nasal tip, dysplastic ears, micrognathia**
Svensson *et al*., 2007	**F**	**developmental delay**	**microcephaly, bitemporal narrowing, high arched palate**
Monfort *et al*., 2008, p2	**M**	**developmental delay, scoliosis**	**high arched palate, dys-plastic ears, micrognathia**
Prontera *et al*., 2009	**M**	**developmental delay, short stature, scoliosis, pectus excavatum**	**scaphocephaly, coarce face, facial asymmetry, long nose, synophris, cleft uvula, micrognathia, dysplastic ears**
Tsai *et al*., 2009, the index patient	**F**	**low birth weight, short stature, developmental delay, hypotonia**	**microcephaly, low-set ears**

Eyes	Upper limbs	Lower limbs	Others
NA	contractures, absent radii, right hand monodactyly, left hand with two digits	contractures, SHFM	ASDII, VSD
downslanting palpebral fissures, hypertelorism, microphthalmia, coloboma, blepharophimosis, myopia	no abnormalities	no abnormalities	brain abnormality, apnoea episodes
astigmatism, hypermetropia, exotropia	tapering fingers	genu valgum, sandal gap	dilatation of brain ventricles, cryptorchidism, inguinal hernia
hypotelorism, ptosis, downslanting palpebral fissures	tapering fingers, clinodactyly V	broad halluces, sandal gap, short toes, hypoplastic phalages	no other abnormalities
strabismus	mild syndactyly II-III and IV-V, clinodactyly V, short middle phalanx V, proximal symphalangism	hallux valgus, proximal and distal symphalangism	no other abnormalities
strabismus	clinodactyly V	syndactyly II-IV	cortico-subcortical atrophy
proptosis	camtodactyly, tapering fingers, clinodactyly V	short metatarsals III-V, sandal gap	dilatation of brain ventricles, inguinal hernia, macroorchidism
no abnormalities	tapering fingers, short middle phalanges II and V, clinodactyly V	duplication of the 1st distal phalanx, broad proximal 1st phalanx, broad 1st metatarsal, hypoplastic/ absent middle phalanges	hearing loss

limb reduction anomalies described as a form of SHFM-monodactylous ectrodactyly (Del Campo *et al*., 1999; Goodman *et al*., 2002; Bijlsma *et al*., 2005). They all carried larger 2q31deletions encompassing the *HOXD* cluster and extending in both centromeric and telomeric directions (Figure 2A). It was proposed that their limb defects were caused by a gene(s) at the new SHFM5 locus, of which *DLX1* and *DLX2* were the best candidates (Goodman *et al*., 2002). However, our data exclude this option, since hemizygosity of the transcripts surrounding the *HOXD* genes at chromosome 2q31.1, but not including the *HOXD* cluster and its regulatory sequences, is not sufficient to produce any abnormal limb development (Patients 4 and 5). In addition, individuals with homozygous *HOXD13* polyalanine tract expansions or compound heterozygotes for *HOXD13* and *HOXA13* alterations present with hand and foot anomalies which partially overlap the severe limb defects in some patients with 2q31.1 deletions (Muragaki *et al*., 1996; Debeer *et al*., 2002; Kuru *et al*., 2004; Horsnell *et al*., 2006). Hence, mainly the *HOXD* haploinsufficiency should explain the more severe skeletal phenotype of these unusual cases.

Interestingly, there are four published patients where different, apparently balanced *de novo* chromosomal rearrangements affecting the 2q31.1 locus were associated with skeletal defects (Spitz *et al*., 2002; Sugawara *et al*., 2002; Dlugaszewska *et al*., 2006). Each of these individuals had a unique phenotype. This ranged from (1) mesomelic dysplasia (Spitz *et al*., 2002; Sugawara *et al*., 2002); through (2) hand brachy-/synpoly-dactyly (patient 1 of Dlugaszewska *et al*. (2006); to (3) severe terminal limb truncations similar to those described as SHFM- monodactylous ectrodactyly (Patient 2 and Patient 3 of Dlugaszewska *et al*. (2006). In all these patients, the chromosome 2q31.1 breakpoints were in close proximity to the *HOXD* cluster and none of the aberrations directly disrupted a known gene (Figure 2A). Therefore, the authors proposed that an abrogated function of centromeric or telomeric *HOXD* regulatory sequences would explain the corresponding preferential anomalies of hands/ feet or forearms/ lower legs (Spitz *et al*., 2002; Sugawara *et al*., 2002; Dlugaszewska *et al*., 2006). However, our survey did not confirm this hypothesis since no segregation of the observed limb defects was detected in patients with different chromosome 2q31.1 deletions (Figure 2A and references (Svensson *et al*., 2007; Pescucci *et al*., 2007; Monfort *et al*., 2008; Prontera *et al*., 2009)). Hence, we suppose that not just simply removing the *HOXD* cluster and/ or its known regulatory sequences (Spitz *et al*., 2003; Zakany *et al*., 2004;

Gonzalez *et al*., 2007; Yamagishi *et al*., 2007), but a more complex, and probably more than one, mechanisms are responsible for this phenotypic variability in these affected individuals (Spitz *et al*., 2002; Sugawara *et al*., 2002; Dlugaszewska *et al*., 2006). The different genetic background is one possibility, such as e.g. (1) the presence of cis- or trans-acting *HOXD* modifiers as mutations/ polymorphisms/ CNVs of other genes involved in the skeletal/ limb development (Debeer *et al*., 2002; Kmita *et al*., 2005; Kmita and Duboule, 2007). Another option is that the acquired new chromosomal landscape or nuclear environment might cause an aberrant/ neomorphic effect upon the *HOXD* function due to (2) a loss of limb specific and adopting of new enhancers/ suppressors (Kleinjan and van Heyningen, 2005; Kleinjan and Lettice, 2008; Spitz and Duboule, 2008). Last but not least, there could be (3) a creation of chimera transcripts (Dlugaszewska *et al*., 2006; Kleinjan and Lettice, 2008; Spitz and Duboule, 2008) or other (4) epigenetic modifications of the chromatin structure which seem to be important for the appropriate spatio-temporal and/ or collinear *HOXD* expression (Tarchini and Duboule, 2006; Morey *et al*., 2007; Montavon *et al*., 2008; Soshnikova and Duboule, 2009). Thus, the effect of one of these factors or the stochastic combination of more than a single event can trigger the observed discrepancy in the abnormal limb morphogenesis.
Finally, patients with hemizygosity of the complete *HOXD* cluster or its regulatory sequences present with a pure *HOXD* genes' haploinsuficiency. This explains their different and less severe limb defects in comparison with those in individuals with *HOXD13* mutations (polyalanine tract extension and point mutations) or small *HOXD9-13* deletions (Goodman *et al*., 1998, 2002; Caronia *et al*., 2003; Johnson *et al*., 2003; Kan *et al*., 2003; Zhao *et al*., 2007; Fantini *et al*., 2009). These clinical observations correlate well with available animal models, functional studies and the principle for *HOXD* functional collinearity (Bruneau *et al*., 2001; Caronia *et al*., 2003; Johnson *et al*., 2003; Zhao *et al*., 2007; Fantini *et al*., 2009; Kuss *et al*., 2009).

Facial dysmorphism
The facial gestalt in the 2q31 microdeletion syndrome is well-defined and clinically identifiable. Individuals that are hemizygous for the 2q31.1 locus involved in the cranio-facial development (FG) share common features as described above (Table 1, Figure2B). Despite this distinctive clinical pattern, it is difficult to assign a single gene within

the defined critical FG region to the observed mental handicap and facial dysmorphism.

Other abnormalities

The observed internal organ and axial skeleton abnormalities, oral clefts, and craniosynostosis cannot be certainly linked to chromosome 2q31.1 region. Some of them, like seizures (Pereira *et al*., 2004; Langer *et al*., 2006) and heart defects (Maas *et al*., 2000), are often referred to up- and down-stream sequences outside the 2q31.1 microdeletion syndrome critical locus. Preliminary data suggest the presence of gene(s) involved in the brain and eye development located at 2q31.1 (Delle Chiaie and Mortier- unpublished data).

In summary, the results of this study provide clear evidence that the limb defects in patients with 2q31 deletions are due only to the *HOXD* cluster hemizygosity. Reinforcing the previous hypothesis for *HOXD* causality, our data unambiguously exclude the possibility for the presence of a separate, *HOXD*-independent SHFM5 locus (Goodman *et al*., 2002). Mechanisms that can explain the observed severe skeletal anomalies in some "atypical" cases are suggested. In addition, a critical region for the common facial phenotype of individuals carrying 2q31 deletions is proposed. Thus, we demonstrate that the 2q31.1 microdeletion is a well-defined and clinically recognizable contiguous gene syndrome mapped to a specific genomic locus. The distinctive combination of limb defects, developmental delay and characteristic facial gestalt is strongly suggestive for the correct diagnosis and appropriate genetic testing.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The available data from the Human Genome Project about the reference Human Genome Sequence and the advent of new, more powerful techniques for genome analysis led to an explosion of often unpredictable findings. In the present project, we exploited all these recent advances in human genetics and related technologies, and we introduced and applied a combination of classical and novel techniques for the genetic analysis of a selected group of patients with variable developmental skeletal and limb defects of unknown aetiology. All individuals were clinically characterized and sub-grouped for subsequent genetic analysis in a stepwise way. Starting from the clinic, going through gene and/ or genomic screening, and returning to the patients' phenotype results in re-grouping for future investigations and the development of a systematic approach for genetic testing in the Clinic. In addition to this "systematic approach" combining classical "forward genetic" and "reverse genetic" methodologies, we exploited data from different electronic sources to explain our findings. Finally, the findings from our studies were used subsequently to complement previous observations. Based on this systematic approach for clinical classification and genetic analysis we were able to detect causal genetic abnormalities in 18.5% of the studied individuals.

In the first part of this thesis classical and new techniques have been applied for molecular characterization of balanced chromosomal rearrangement (*de novo* translocation and inversion) associated with specific skeletal phenotypes (MSD and KFA). The precise breakpoint mapping revealed neither directly disrupted known genes by the chromosomal aberrations nor abnormal expression in EBV-cell lines of selected candidate genes in the vicinity. In addition, recent publications reported on chromosomal deletions encompassing all four breakpoint described in this thesis (Barber *et al*., 2005; Thienpont *et al*., 2005; Caselli *et al*., 2007; Brichard *et al*., 2008). However, no skeletal defects were detected in these patients. Therefore, haploinsufficiency of gene expression up- and down-stream from the breakpoints due to position effect could be ruled out.

In more than 40% of individuals, who are carriers of balanced chromosomal aberrations, there is no gene disrupted by the chromosomal rearrangement (Vandeweyer and Kooy, 2009). This is probably an underestimation since many similar cases probably remain unpublished. The absence of plausible candidate gene(s) in the vicinity represents currently a real challenge. There is no doubt that at least in certain of these cases the observed chromosomal aberrations

probably contribute to the phenotypic abnormalities and only technology limitations restrict our ability to prove this.
The second part of the thesis illustrates the relevance of the array CGH analysis in patients with congenital skeletal defects, particularly LRDs. Based on this approach we were able to define 10q24q25 microduplication/ triplication as a molecular cause of Distal Limb Deficiency Micrognathia Syndrome. We showed that the same chromosomal aberration is associated with syndromic forms of SHFM thus demonstrating the broad clinical spectrum of SHFM3. Our findings confirm the existence of DLDMS and make a molecular verification of the clinical diagnosis with all its important consequences for genetic counselling available. In addition, they provide an important contribution to understanding the underling molecular mechanism of disturbed limb development. The currently accepted cause of disturbed *Dactylyn* (*FBXW4*) function in the pathogenesis of SHFM3 seems to be insufficient to explain the observed limb and non-skeletal defects. Several recent publications suggest that *FBXW4* is probably a "bystander" rather than a real cause of the phenotype (Kano *et al*., 2005, 2007; Kikuta *et al*., 2007; Friedli *et al*., 2008). For the first time we report that the severity of the phenotype is not linked to the extent of the detected duplications but is influenced by the increased genomic copy numbers at the SHFM3 locus. This finding, together with the other molecular studies, supports the hypothesis that an altered balance and interactions between regulatory elements and their target genes could more satisfactorily explain the complex SHFM3 phenotype. Logically, the phenotypic variability could be a result of the affected function of more than one gene. Thus, *FGF8* could be the cause of the observed limb and facial abnormalities, whereas *PITX3* and *PAX2* could be responsible for the ocular and kidney defects. *PITX3*, together with *PITX1*and *PITX2*, belongs to a novel family of a paired-like class of homeodomain *PTX* transcription factors. *PITX3* mutations are associated with congenital glaucoma, anterior eye segment mesenchymal dysgenesis and myopia (Sakazume *et al*., 2007). Similarly, *PAX2* is currently the only known gene causing oligomeganephronia/ renal hypoplasia in man (Salomon *et al*., 2001). Both *PAX2* and *PITX3* are in close proximity to the detected 10q24 duplication/ triplication in syndromic forms of SHFM3. Interestingly *FGF8* is also involved in the eye and kidney development. Therefore the combined effect of several genes under the control of stochastic events could determine the highly variable final phenotype, but this hypothesis needs further research. Good options for analysis provide the availability of different strains of *FGF*

knock-out mice. Future collaboration with a research group (Spitz-Heidelberg, Germany) who was able to generate a mutant mouse corresponding to the detected 10q24 duplication in patients with SHFM3 will allow more precise investigation of the intimate molecular mechanisms underlying the abnormal mouse and human development. A presumptive outcome is the selection of candidate gene(s). Therefore, the collection of samples from SHFM3 families with positive linkage but no associated 10q24 duplication/ triplication is an important issue for further candidate gene validation.

Another original finding was the detection of CNVs within the *GREMLIN1-FORMIN1* region in individuals with two new syndromes. Since a long time, this locus has been known to be implicated in mouse limb development. Now, for the first time we were able to demonstrate its importance in human limb development and pathology. We were able to identify a homozygous *FMN1* deletion in a patient with Oligosyndactyly, Radio-Ulnar synostosis, Hearing loss and Renal defects, and a duplication of *GREM1* and *FMN1* in an individual with isolated Cenani-Lenz-like oligosyndactyly. Both phenotypes delineate new syndromes within the Cenani-Lenz oligosyndactyly spectrum. In addition, the isolated Cenani-Lenz-like oligosyndactyly is a good candidate for a new genomic disorder. Unfortunately no additional *GREM1* and *FMN1* abnormalities were detected within a group of patients with phenotypes similar to those of the index individuals. Therefore extending the clinical criteria for genetic analysis should unravel the real clinical spectrum of these new syndromes.Although there are corresponding animal models with a reminiscent phenotype to those of the patients with *GREM1* and *FMN1* rearrangements, it is not clear to what extent each of these two genes contributes to the observed phenotypic characteristics. An option for future research would be to investigate the effects of the detected *FMN1* homozygous deletion and *FMN1* over-expression in transgenic mouse and chick.

Interestingly, during the primary clinical selection all individuals with detected chromosome 10q24q25 (SHFM3 locus) and 15q13.3 (*GREM1-FMN1* locus) rearrangements were sub-grouped together for further genetic analysis. Based on our current knowledge, the phenotype of the patients with 15q13.3 deletions/ duplications affecting the *GREM1-FMN1* locus is the result of abrogated m-e crosstalk between the ZPA and the AER. The final effect will be a disturbed FGF function (mainly *FGF8*/ *FGF4*) within the AER. This will subsequently lead to an abnormal limb development. As discussed above, *FGF8* is one of the major candidates to cause the

phenotype in syndromic and non-syndromic SHFM3 patients. In *dactylaplasia* mice there is an abnormal AER expression profile of the *FGF8* gene. This could be used as indirect evidence for *FGF8* involvement in the SHFM3 clinical features. Vice versa it demonstrates the correct systematic approach for clinical management, evaluation and genetic analysis used in this study. In this way, the appropriate and systematic clinical selection and molecular testing will allow identification of genes involved in developmental pathways, causing gene family disorders. The gathered information will further improve our understanding for the genotype/ phenotype relationships in the group of developmental skeletal disorders. This process will certainly be enhanced with future implementation of database mining tools for gene prioritisation (e.g. ENDEAVOUR) in positional candidate gene approaches for gene identification (e.g. breakpoint mapping and array CGH) (Aerts *et al*., 2006).

In this project, we also re-evaluated the clinical phenotype in patients with the 2q31 deletion syndrome and precisely redefined the genotype/phenotype correlations in an attempt to characterize more accurately the SHFM5 locus. We were able to demonstrate that only the HOXD genes are involved in the pathogenesis of limb defects in individuals with 2q31 deletions. Furthermore, we delineated the critical loci responsible for the facial gestalt and skeletal abnormalities suggesting a clinically recognizable 2q31.1 microdeletion syndrome. Affected individuals have variable hand and foot anomalies caused by "pure" *HOXD* haplo-insufficiency or hemizygosity of *HOXD* regulatory sequences. Therefore it is interesting to further investigate whether mutations (point mutations or genomic rearrangements) in these *HOXD* regulatory elements are responsible for the similar skeletal defects in patients with different forms of brachydactyly (e.g. brachydactyly type A2 and A3) or symphalangism. Characterization of the deletion size in more individuals with 2q31.1 deletions will differentiate whether a single gene can be assigned to specific facial characteristics or whether the delineated typical facial gestalt is a result of a continuous gene syndrome.

Finally, a substantial part of the studied individuals with developmental skeletal and limb defects in this project (patients with Holt-Oram-like phenotype and no TBX5 mutations/ deletions and patients with normal 1MB array CGH analysis) are still waiting for a molecular explanation for their phenotype. This group represents a suitable population for future CNV screening at higher resolution. A more daunting task will be to explain some more complex phenotypes. For example, why do some 2q31 deletion patients have severe limb

defects like SHFM/ monodactylous ectrodactyly with absent long bones of the forearm?

Therefore, the development and implementation of new techniques and systems biology approaches to study complex developmental disorders is a hallmark strategy to improve our knowledge about unresolved questions. The new generation sequencing and cell reprogramming (induced pluripotent stem cells) combined with the data of projects investigating the functional integrity of the Human Genome is envisaged. Their implication in genetic studies of congenital anomalies due to multifaceted errors in the development will certainly challenge our current single-gene way of thinking and will shift many basic paradigms in genetics and medicine.

The next generation sequencing represents a variety of new strategies that allow the production of large-scale qualitative and quantitative information about any type of nucleic acid in a given genome at an unprecedented high throughput level. As its laboratory price dramatically diminished the last one-two years, it has become possible for more research projects to exploit this new technology. With the promised cost of 1000$ per genome, the next generation sequencing will certainly become routinely available in clinical diagnosis (Tucker *et al*., 2009; Metzker, 2010). An important application is whole genome screening for discovery of novel mutations in rare genetic disorders. For several decades, the discovery of new genes underlying Mendelian disorders was based on positional cloning, physical mapping or candidate-gene sequencing. However, for rare phenotypes or for disorders with a high level of genetic heterogeneity these approaches failed to be efficient. Now, whole genome sequencing of the genomes of a few unrelated patients or even small nuclear families combined with filtering methods for selection of the most likely causal genome variants will be a straightforward and powerful method for gene "hunting". As the majority of the currently detected mutations implicated in genetic disorders affect protein coding sequences, an alternative is the targeted sequencing of the exome (the protein coding part of the genome) which constitutes as much as 5% of the whole genome. Interestingly, a model *for the recently published proof-of-concept study was Miller syndrom*e, a form of acrofacial dysostosis characterized with postaxial RLDs (Ng *et al*., 2010). Transcriptome sequencing, proteomic or even epigenetic data could additionally enhance the efficacy of this strategy. Another opportunity is the combined exploitation of linkage analysis (including homozygosity

mapping) with next generation sequencing of the obtained loci under linkage (Nikopoulos *et al*., 2010). This will substantially reduce the project costs and will allow the inclusion in the sequencing assay not only coding exons but also non-coding/ micro-RNAs, highly conserved non-coding elements or even the entire linkage interval. In addition, data from whole genome screening can be used to search modifiers of the phenotype or for parallel sequencing of entire pathways implicated in specific developmental processes and heterogeneous genetic syndromes/ syndrome families.

The possibility for cell reprogramming to an embryonic stage and the creation of pluripotent cell lineages is another technique providing new perspectives to study normal and abnormal early human development. These induced pluripotent stem cells (e.g. fibroblast and keratinocyte derived) share a highly similar expression and epigenetic status with embryonic stem cells. In theory, this state should provide them a full developmental potential. Thus, they will have the characteristics to self-renew indefinitely and can give rise to all cell types in the human body, but are generated via reprogramming of somatic cells through the forced expression of a few key transcription factors as OCT4, SOX2, KLF4 and c-MYC. This technique has been used with success to generate an entire mouse by induced pluripotent stem cells (Boland *et al*., 2009; Zhao *et al*., 2009). Similarly, the abnormal genetic background in patient's cells was genetically corrected and subsequently these genetically modified cells were reprogrammed (Raya *et al*., 2010). These experiments illustrate the promising use of this new technology in regenerative medicine, gene therapy and disease modelling. In this way, limitations in the study of the early limb and skeletal development discussed above can be circumvented. For example the limit of obtaining appropriate patients' samples will not restrict the investigation of tissue specific spatio-temporal expression of developmental genes, position effect, point mutations affecting regulatory sequences or teratogen mechanisms (Nishikawa *et al*., 2008; Kiskinis and Eggan, 2010). In addition, patient derived induced pluripotent stem cells can be used in primary screenings for selection of new disease gene candidates (e.g. patients with Holt-Oram syndrome like phenotype and no *TBX5* mutations/intragenic deletions).
In conclusion, exploitation of the capabilities of the new generation sequencing and induced pluripotent stem cells represents our vision

for future research in the field of limb formation and skeletogenesis. This new approach, recently referred as systems biomedicine, allows studying the limb development as integrated and complex developmental machinery, (Liu, 2009).

APPENDICES

Appendix A. International Nosology and Classification of Genetic Skeletal Disorders—2006 revision (Adapted from Rimoin *et al.* 2007)

1. FGFR3 group
2. Type II collagen group
3. Type XI collagen group
4. Sulfation disorders group
5. Perlecan group
6. Filamin group
7. Short-rib dysplasia (SRP) (with or without polydactyly) group
8. Multiple epiphyseal dysplasias and pseudoachondroplasia group
9. Metaphyseal dysplasias
10. Spondylometaphyseal dysplasias (SMD)
11. Spondylo-epi(-meta)physeal dysplasias (SE(M)D)
12. Severe spondylodysplastic dysplasias
13. Moderate spondylodysplastic dysplasias (brachyolmias)
14. Acromelic dysplasias
15. Acromesomelic dysplasias
16. Mesomelic and rhizomesomelic dysplasias
17. Bent bones dysplasias
18. Slender bone dysplasias
19. Dysplasias with multiple joint dislocations
20. Chondrodysplasia punctata (CDP) group
21. Neonatal osteosclerotic dysplasias
22. Increased bone density group (without modification of bone shape)
23. Increased bone density group with metaphyseal and/or diaphyseal involvement
24. Decreased bone density group
25. Defective mineralization group
26. Lysosomal storage diseases with skeletal involvement (dysostosis multiplex group)
27. Osteolysis group
28. Disorganized development of skeletal components group
29. Cleidocranial dysplasia group
30. Craniosynostosis syndromes and other cranial ossification disorders
31. Dysostoses with predominant craniofacial involvement
32. Dysostoses with predominant vertebral and costal involvement

33. Patellar dysostoses
34. Brachydactylies (with or without extraskeletal manifestations)
35. Limb hypoplasia–reduction defects group
36. Polydactyly-syndactyly-triphalangism group
37. Defects in joint formation and synostoses

Appendix B. Molecular – Pathogenesis Classification of Skeletal Dysplasias (Adapted from Rimoin *et al*. 2007)

Defects in extracellular structural proteins

COL1A1, COL1A2: Osteogenesis imperfecta

COL2A1: achondrogenesis type 2, hypochondrogenesis, SEDC, SEMD, Kniest, Stickler, Familial osteoarthritis

COL9A1, COL9A2, COL9A3: Multiple epiphyseal dysplasia (MED)

COL10A1: Metaphyseal dysplasia Schmid

COL11A1, COL11A2: Oto-spondylo-megaepiphyseal dysplasia (OSMED); Stickler (variant), Marshall syndrome

COMP: Pseudoachondroplasia, MED

MATN3: Multiple epiphyseal dysplasia (MED)

Perlecan: Schwartz-Jampel type 1; dyssegmental dysplasia

Defects in metabolic pathways

TNSALP (tissue non-specific alkaline phosphatase): Hypophosphatasia (several)

ANKH (pyrophosphate transporter): Craniometaphyseal dysplasia

DTDST (diastrophic dysplasia sulfate transporter): achondrogenesis 1B, atelosteogenesis 2, diastrophic dysplasia, autosomal recessive MED (rMED)

PAPSS2 (phosphoadenosine-phosphosulfate-synthase 2): Spondylo-epi-metaphyseal dysplasia Pakistani type

CHST3 (chondroitin 6-O-sulfotransferase-1): SEMD Omani type

Defects in folding, processing, transport, and degradation of macromolecules

Sedlin (unknown function): X-linked Spondyloepiphyseal dysplasia (SED-XL)

Cathepsin K (lysosomal proteinase): Pycnodysostosis

Lysosomal acid hydrolases and transporters: Mucopolysaccharidoses, oligosaccharidoses, glycoproteinoses

Targeting system for lysosomal enzymes (GlcNAc-1-phosphotransferase): Mucolipidosis II & III

MMP2 (matrix metalloproteinase 2): Torg type osteolysis

Tubulin chaperonin E: Kenney-Caffey and Sanjod-Sakati syndromes (TBCE)

EXT1, EXT2: Multiple exostoses syndrome types 1&2

SH3BP2 (c-*Abl*-binding protein): Cherubism

Defects in hormones, growth factors, receptors, and signal transduction

FGFR1: Craniosynostosis syndromes (Pfeiffer)

FGFR2: Craniosynostosis syndromes (Apert, Crouzon, Pfeiffer)
FGFR3: Thanatophoric dysplasia, achondroplasia, hypochondroplasia, SADDAN; craniosynostosis syndromes
ROR-2 ("orphan receptor tyrosine kinase"): AR-Robinow syndrome, AD-Brachydactyly type B
PTH/PTHrP receptor: (activating mutations) Metaphyseal dysplasia Jansen, (inactivating mutation) Blomstrand lethal dysplasia
GNAS1 (stimulatory Gs _ protein of adenylate cyclase): Pseudohypoparathyroidism (Albright Hereditary Osteodystrophy) with constitutional haploinsufficiency mutations, McCune–Albright syndrome with somatic mosaicism for activating mutations

Defects in nuclear proteins and transcription factors

SOX9: Campomelic dysplasia
TRPS1 (zinc-finger gene): Tricho-Rhino-Phalangeal syndromes
CBFA-1 (runt-type transcription factor): Cleidocranial dysplasia
LXM1B (LIM homeodomain protein): Nail-patella syndrome
SHOX (short stature—homeobox gene): Leri–Weill dyschondrosteosis, Turner syndrome
EVC (Leucine-zipper gene): AR-Chondroectodermal dysplasia (Ellis-van Creveld), AD-Weyers acrodental dysostosis

Defects in RNA processing and metabolism

RMRP: Cartilage-hair-hypoplasia
SDDS–Schwachman–Diamond syndrome

Defects in cytoskeletal proteins

Filamin (actin binding proteins):
Filamin A: Otopaltodigfital syndromes I & II; frontometaphyseal dysplasia; Melnick-Needles
Filamin B: Spondylocarpotarsal syndrome; Larsen syndrome; Atelosteogenesis I/III; Boomerang dysplasia

Responsible gene identified, but function unknown

Dymeclin: Dyggve–Melchior–Clausen syndrome; Smith–McCort syndrome

Appendix C. Mesomelic dysplasias in the International Nosology and Classification of Genetic Skeletal Disorders – 2006 (Adapted from Superti-Furga *et al*. 2007).

Name of Disorder	Inheritance	MIM No.	Locus	Gene	Protein	MIM No.	Notes
16. Mesomelic and rhizo-mesomelic dysplasias							
Dyschondrosteosis (Leri-Weill)	Pseudo-AD	127300	Xpter-p22.32 (pseudo-autosomal)	SHOX	Short stature—homeobox gene	312865	Includes Reinhardt-Pfeiffer dysplasia, MIM 191400
Langer type (homozygous dyschondrosteosis)	Pseudo-AR	249700	Xpter-p22.32 (pseudo-autosomal)	SHOX	Short stature—homeobox gene	312865	
Robinow syndrome, recessive type	AR	268310	9q22	ROR2	Receptor tyrosine kinase-like orphan receptor 2	602337	Includes previous COVESDEM (costo-vertebral segmentation defect with mesomelia); see also brachydactyly type B, Group 34
Robinow syndrome, dominant type	AD	180700					
Mesomelic dysplasia, Kantaputra type	AD	156232	2q24-32				
Mesomelic dysplasia, Nievergelt type	AD	163400					
Mesomelic dysplasia, Kozlowski-Reardon type	AR	249710					
Mesomelic dysplasia with acral synostoses (Verloes-David-Pfeiffer type)	AD	600383					
Mesomelic dysplasia, Savarirayan type (Triangular Tibia-Fibular Aplasia)	SP	605274					Possibly related to Nievergelt dysplasia
Omodysplasia, dominant type	AD	164745					
Omodysplasia, recessive type	AR	108721					

Appendix D. KFA classifications and associated anomalies (Adapted from Giampietro *et al*. 2009).

Reference	Cervical fusion abnormalities	Thoracic fusion abnormalities	Lumbar fusion abnormalities	Other malformations	Inheritance
Klippel[92]	Short neck, low posterior hairline. Absence of cervical vertebrae				Sporadic
Feil[93]	Group I: massive fusion of many vertebrae Group II: fusion of one or two cervical interspaces Group III: cervical	Groups I and III	Group III	Sprengel deformity Type II (failure of proper scapular descent)	
Heisenger[94]	Type 1: C2−C3 fusion with occipitilization of the atlas Type II: Long cervical fusion with an abnormal occipitocervical junction Type III: Two blocked vertebral segments with a single open interspace				
Clarke[95]	KF1: C1 fusion is the most rostral	NA	NA	KF1-cardiac, urogenital, hearing, craniofacial, limb, digital, ocular defects	KF1: Autosomal recessive
	KF2: C2-C3 fusion is dominant and most rostral			KF2-craniofacial, hearing, otolaryngeal, skeletal and limb defects	KF2: Autosomal dominant
	KF3: C3 (C2–3 or C3–4) most rostral fusion. Isolated fusions			KF3-craniofacial abnormalities	KF3: Autosomal recessive and autosomal dominant
				KF4-Wildervank syndrome (Duane retraction syndrome, characterized by narrowed palpable fissure, globe retraction and failure of abduction of eye + sensorineural hearing loss)	KF4: X linked dominant
Manaligod[96]	Cervical fusion	Thoracic fusion (isolated)			
Samartzis[9]	Type 1: Single congenital fused cervical segment Type II: Multiple noncontiguous congenitally fused segments Type III: Multiple contiguous, congenitally fused cervical segments	NA	NA		

Appendix E. Hypoplasia-reduction limb defects (Adapted from Superti-Furga *et al*. 2007).

Name of Disorder	Inheritance	MIM No.	Locus	Gene	Protein	MIM No.	Notes
35. Limb hypoplasia-reduction defects group							
Acheiropodia	AR	200500	7q36	LMBR1	Putative receptor protein	605522	Partial LMBR1 deletion affecting expression of Sonic Hedgehog (SHH) gene
De Lange Syndrome	AD	122470	5p13.1	NIPBL	Nipped-B-like	608667	
Fanconi anemia	AR	227650	(several)	(several)			Several complementation groups and genes
Holt–Oram syndrome	AD	142900	12q24.1	TBX5	T-box gene 5	601620	
Okihiro syndrome (Duane-Radial Ray anomaly)	AD	607323	20q13	SALL4	SAL-like 4	607343	
Roberts Syndrome	AR	268300	8p21.1	ESCO2	Homolog of Establishment of Cohesion-2	609353	
Tetra-amelia	AR	273395	17q21	WNT3	Wingless-type MMTV integration site family, member 3	165330	
Ulnar-mammary syndrome	AD	181450	12q24.1	TBX3	T-box gene 3	601621	
Ankyloblepharon-Ectodermal dysplasia-Cleft lip/palate (AEC)	AD	106260	3q27	P63 (TP63)	Tumor Protein p63	603273	
Ectrodactyly-ectodermal dysplasia cleft-palate syndrome type 3 (EEC3)	AD	604292	3q27	P63 (TP63)	Tumor Protein p63	603273	
Ectrodactyly-ectodermal dysplasia cleft-palate syndrome type 1 (EEC1)	AD	129900	7q11.2-12.3				
Ectrodactyly-ectodermal dysplasia cleft-palate syndrome type 2 (EEC2)	AD	602077	Chr.19				
Ectrodactyly-ectodermal dysplasia-macular dystrophy syndrome (EEM)	AR	225280	16q22	CDH3	Cadherin 3	114021	
Limb-mammary syndrome (including ADULT syndrome)	AD	603273	3q27	P63 (TP63)	Tumor Protein p63	603273	
Split Hand-Foot malformation, isolated form, type 4 (SHFM4)	AD	605289	3q27	P63 (TP63)	Tumor Protein p63	603273	
Split Hand-Foot malformation, isolated form, type 1 (SHFM1)	AD	183600	7q21.3-22.1				
Split Hand-Foot Malformation, isolated form, type 2 (SHFM2)	XL	313350	Xq26				
Split Hand-Foot malformation, isolated form, type 3 (SHFM3)	AD	600095	10q24	Dactylin	Dactylin	608071	
Split Hand-Foot malformation, isolated form, type 5 (SHFM5)	AD	606708	2q31				
Split Hand-Foot malformation with tibial hypoplasia	AD	119100					
Adams–Oliver syndrome	AD	100300					
Al-Awadi Raas-Rothschild limb-pelvis hypoplasia-aplasia	AR	276820					
Femoral hypoplasia-Unusual facies syndrome	SP/AD?	134780					Syndromic status uncertain
Femur–Fibula–Ulna syndrome	SP?	228200					
Fuhrmann syndrome	AR	228930					
Hanhart syndrome (Hypoglossia-hypodactylia)	AD	103300					
Scapulo-iliac dysplasia (Kosenow)	AD	169550					
Thrombocytopenia-Absent Radius (TAR)	AR?/AD?	274000					
See also CHILD in Group 20							

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PROFESSIONAL CAREER

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University Education

1987-1993	Medical student, Faculty of Medicine, Higher Medical Institute of Sofia, Bulgaria Diploma obtained- Master Physician

Post-graduate Education

1995-2002	Resident in Paediatrics, Faculty of Medicine, Department of Paediatrics, Medical University Sofia, Bulgaria Diploma obtained- Certificate of Specialist in Paediatrics
2004	Pre-Doctoral program in Human Genetics, Centre for Human Genetics, Faculty of Medicine, Department of Human Genetics, Catholic University of Leuven, Belgium Diploma obtained- Certificate of Medical Sciences

Professional career

1994-2000	Registrar/ Clinical fellow in Paediatrics, Department of Paediatric, Section of Paediatric and Clinical Genetics, University Children's Hospital, Medical University Sofia, Bulgaria
2000-2005	Assistant Professor in Paediatrics, Department of Paediatrics, Section of Paediatric and Clinical Genetics, University Children's Hospital, Medical University Sofia, Bulgaria
2004-present	Doctoral student, Centre for Human Genetics, Faculty of Medicine, Department of Human Genetics, Catholic University of Leuven, Belgium

Academic/Research experience

Medical University Sofia, Bulgaria (1994-2005)

- As Assistant Professor in Paediatrics- teaching of under-/post-graduated medical students in Paediatrics and Dysmorphology
- Prevalence, clinical assessment, genetics and management of skeletal dysplasias and congenital skeletal anomalies
- recombinant human Growth Hormone treatment in Clinical Genetics (Turner, Noonan, Prader-Willi syndromes, skeletal dysplasias)
- Enzyme replacement therapy of patients with Gaucher and Fabry diseases;
- Epidemiology and genetics of birth defects (Sofia Registry of Congenital Anomalies- regular member of EUROCAT)

Catholic University of Leuven, Belgium (2004-2010)

- Genetic studies in developmental skeletal and limb defects (Doctoral Thesis)
- CGH array screening for copy number variations in patients with metopic cranio-synostosis
- Homozygosity mapping and linkage analysis

Additional courses and graduated classes

- 1st Focus Course: Protein Glycosylation in Health and Disease, organized by EUROGLYCAN and Orphan Europe Academy, Catania, Italy (1-2 April 2003)
- The 4th Course in Clinical Genetics: From Embryology to Dysmorphology, organized by European Genetic Foundation, Science City Tunis, Tunisia (21-24 November 2004)
- Systems of Genetic Analysis, mandatory course for Doctoral students, organized by Department of Human Genetics, Catholic University Leuven (10 January – 21 February 2005)
- Understanding Medical Statistics in Clinical Trials, Course for Doctoral students in biomedical sciences, organized by Biostatistics Centre, Catholic University of Leuven (November 2006- April 2007)
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- Basic gene mapping course: Linkage and Association studies, organized by Baylor College of Medicine and Max Delbruck Centrum, Berlin, Germany (7-11July 2008)
- Introduction to R: basic course, organized by ICTS, Catholic University of Leuven (23-26 February 2009)

Awards and Fellowships

- 1 year and 2 months Clinical/ Research fellowship Award from the Government of Flanders, Belgium, administrated by Ministry of Science and Education of Bulgaria. This is awarded to two Bulgarian Academic specialists per year from all branches of the Science. The award was taken at the Centre for Human Genetics, University Hospital Gasthuisber, Catholic University of Leuven, Belgium for clinical specialization in Medical Genetics (2002-2003)
- 4 year Doctoral scholarship of the Catholic University of Leuven, Belgium (2004-2008)

LIST OF PUBLICATIONS

International peer-review articles

1. **Dimitrov B**, Devriendt K, Maas NMC, Vermeesch JR, Popova A, Simeonov E and Fryns JP. Mesomelic form of chondrodysplasia and congenital glaucoma associated with de novo translocation (13; 18)(q14; q23). Genet Couns 2004;15:191-197.
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5. Stefanova M, Zemke K, **Dimitrov B**, Has C, Kern JS, Bruckner-Tuderman L, Kutsche K. Disruption of ERBB2IP is not associated with dystrophic epidermolysis bullosa in both father and son carrying a balanced 5;13 translocation. J Invest Dermatol 2005;125:700-704.
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9. Kirchhoff M, Bisgaard AM, Stoeva R**, Dimitrov B**, Gillessen-Kaesbach G, Fryns JP, Rose H, Grozdanova L, Ivanov I, Keymolen K, Fagerberg C, Tranebjaerg L, Skovby F, Stefanova M. Phenotype and 244 array-CGH characterization of chromosome 13q deletions: an update of the phenotypic map of 13q21.1-qter. Am J Med Genet 2009;149:894-905.
10. **Dimitrov BI**, de Ravel T, Van Driessche J, de Die-Smulders C, Toutain A, Vermeesch JR, Fryns JP, Devriendt K, Debeer P. Distal limb deficiencies, micrognathia syndrome (OMIM 246560) and syndromic forms of split hand foot malformation (SHFM) are caused by chromosome 10q genomic rearrangements. J Med Genet 2010;47:103-111.
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12. **Dimitrov BI**, Balikova I, de Ravel T, Van Esch H, De Smedt M, Baten E, Vermeesch JR, Bradinova I, Simeonov E, Devriendt K, Fryns JP and Debeer P. 2q31.1 microdeletion syndrome-redefining the associated clinical phenotype. 2010 (Manuscript submitted).

Other publications

1. Симеонов Е, **Димитров Б**, Захариев Д. Лечение на скелетните дисплазии с растежен хормон [Simeonov E, **Dimitrov B**, Zahariev D. Treatment of skeletal dysplasias with recombinant human Growth Hormon. Pediatria- original language Bulgarian]. Педиатрия, 2/1998:16-19.
2. Симеонов Е, Сталев В, **Димитров Б**, Рачева Ж. Синдром на Roberts (Псевдоталидомидов синдром, SC фокомелия синдром) [Simeonov E, Stalev V, **Dimitrov B**, Racheva J. Clinical dysmorphology- Roberts syndrome (Pseudothalidomide syndrome, SC Phocomelia syndrome). Pediatria- original language Bulgarian]. Педиатрия, 2/1998:35-36.

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